STUDIES ON THE SPERMATOZOA OF THE RAM WITH SPECIAL REFERENCE TO THE EFFECTS OF DEEP FREEZING

VOLUME I

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This thesis is dedicated in loving memory to my late father, and also to those who are now closest to me, my dear mother, my beloved wife, and children, Ali, Shatha and Nahla.

I declare that the content of this thesis is my own work and that it has not been presented to any university other than to the University of Edinburgh.

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III - SUMMARY

This thesis describes studies on the spermatozoa of the ram carried out with a view to determining the effects of deep freezing which lead to poor fertility following artificial insemination.

The electro-ejaculator was employed for semen collection over 20 months and 80 samples out of 114 collections were processed.

Motility of spermatozoa was assessed by scoring mass activity in raw semen samples and by estimation of the percentage of motile spermatozoa in raw and processed samples.

Percentages of live spermatozoa and their general morphology were studied in eosin-nigrosin stained smears and acrosomal defects in eosin fast green FCF stained smears. Spermatozoal morphology was also studied with the electron microscope.

Generally the various parameters of the raw semen were within or close to the standard ranges and they varied with season, best semen being collected in the autumn.

When semen was frozen by a standard technique there was a continuous reduction in the spermatozoal viability, especially motility, associated with an increase in morphological deterioration of the spermatozoa, especially of their acrosomes, following each stage. At the same time variation in spermatozoal viability and morphology existed between different samples.

Various modifications of the freezing technique were tried. On inclusion of 2-8% glycerol or dimethyl sulphoxide (DMSO), or their combinations at different levels in an egg yolk and lactose diluent the results respectively showed that 4% glycerol, 3% DMSO and 2% glycerol with 1.5% DMSO were the optima.

The effects on spermatozoa of various equilibration times (0.5 - 24.0 hours), dilution rates (1:1 - 1:10), thawing media (sodium chloride or citrate with or without lactose in solution at 37°C or frozen pellet at -196°C) and thawing temperatures (0°C - 100°C) were studied using both 4% glycerol or 3% DMSO as the cryoprotective in the diluents. The results showed that equilibration of 3.0 hours with glycerol, and of 1.5 hours with DMSO, and dilution rates of 1:4 and thawing directly in a dry test tube at 37°C - 100°C, irrespective of the cryoprotective, were the optima.

In addition, various methods of dilution (dropping or direct at 4°C or 20°C or their combination) and different egg yolk levels (25 or 50%) with or without sodium citrate (3%) were used. The results indicated that equilibration at 20°C, irrespective of method of dilution led to a high death rate of ram spermatozoa but provided surviving spermatozoa some resistance against cold shock during freezing. Direct addition of the diluent irrespective of the equilibration temperature was satisfactory, but the addition of the diluent by dropping for 0.5 hour at 20°C followed by 1.0 hour equilibration at 4°C was superior.

Increasing the egg yolk level from 25 to 50%, with or without sodium citrate in 4% glycerol containing diluent was harmful which might be the result of binding up of glycerol.

Prompt dilution of the raw semen after collection and the avoidance of temperature fluctuation between stages of freezing and when sampling for thawing were tried and the results showed an enhanced resistance to cold shock and an improvement in spermatozoal motility when thawed after 24 hours storage at -196°C. However, longer storage

was still deleterious, and the percentage of motile spermatozoa fell to 40%.

The post-thawing life span and morphological changes of the ram spermatozoa were evaluated following thawing at $0^{o}C$ - $100^{o}C$ and incubation at $39^{o}C$ for 0, 3.0 and 6.0 hours. The results showed that the life span of the frozen-thawed ram spermatozoa was short (around 3 hours) and their acrosomal defects increased progressively as the incubation time increased.

The fertilizing efficiency of frozen semen stored for 42 - 46 days was tried on 28 ewes, but the results showed that the low post-thawing motility and short survival time which pertained were not adequate to produce pregnancy.

IV INTRODUCTION

Artificial insemination (A.I.) of sheep and cattle with fresh and diluted semen was first introduced by Ivanoff (1928) in U.S.S.R. Since that time extensive research has been carried out on the efficiency of A.I. with the conclusion that while fertility is generally equal to or higher than fertility from natural service in cattle, in sheep the results are less successful especially after dilution or storage at $5^{o}C$ - $10^{o}C$ (Eldjarn, 1946, and Aamdal and Hogset, 1955). Improved fertility, however, could be obtained in sheep by increasing the number of inseminations during oestrus (Bonadonna, 1942).

The indefinite preservation of living tissues by freezing has been the goal of the research workers. The problems of dehydration as well as the crystallization of water contents of the living cells to some extent were resolved by the addition of ethylene glycol as discovered by Luyet and Harting (1941) in freezing the eelworm or the addition of glycerol as discovered by Rostand (1946) in freezing frog spermatozoa and Polge, Smith and Parkes (1949) in freezing fowl spermatozoa. Shortly after that, successful freezing of bull semen was achieved by Smith and Polge (1950a). The efficiency of such frozen semen was tested by Stewart (1951) and only one calf was born among five inseminations. Later on Polge and Rowson (1952a &b) obtained a fluctuating fertility rate ranging between 0 - 79%.

However, nowadays frozen bull semen is widely used for routine artificial insemination on a commercial scale, having expanded progressively since 1954, when, Waterloo Cattle Breeding Association of Waterloo, Guelph, Ontario, became the first breeding organization to use frozen bull semen (quoted by Perry, 1968).

But workers are still interested in frozen semen of farm animals other than cattle such as horse, pig, and sheep. Emmens and Blackshaw (1950) froze ram spermatozoa according to the methods of Polge et al. (1949) and Smith and Polge (1950a) but did not obtain satisfactory post thawing survival (5-10%) in spite of their varying the kinds of sugar and glycerol percentages in the diluent. In 1956 Markovic obtained 60% motile spermatozoa after freezing ram semen, using a diluent containing sugar (arabinose or glucose), egg yolk and glycerol, and equilibration time of 6-14 hours.

Many investigators, later on, achieved similar success in freezing ram semen, by choosing the proper diluent, dilution rate, equilibration time, freezing techniques, thawing temperatures and thawing media, as reported in the reviews of Emmens and Robinson (1962), Lunca (1964), Terrill (1968) and Nishikawa (1964 & 1972). However, the conception rate varied considerably with individual workers and was generally too low for wide practical application. Smirnov (1951) claimed that in 1949 he obtained 11 lambs from 19 ewes inseminated with frozen ram semen. Blackshaw and Emmens (1953) and Emmens and Blackshaw (1955) reported lambing rates of only 10% and 5% respectively with semen of very good post-thawing revival and motility.

Higher conception rates have been obtained by a few investigators, but mostly either by using more than one insemination during the single heat period (First, Sevinge and Henneman, 1961), or by modification of the insemination technique such as intra uterine insemination by laparotomy (Fraser, 1968).

The problem of the low fertilizing capability of the frozen ram spermatozoa, in spite of their reasonable post thawing activity, remains unsolved.

It is well known that the sperm acrosome plays a major role in the fertilization process (Austin, 1960; Bedford, 1963a and Murdoch and White, 1968). The first morphological investigations of the acrosome were carried out under the light microscope in 1945 (Blom, 1945) and the electron microscope in 1957 (Walton, 1957).

The spermatozoal acrosome was found to be the actual site of the freezing damage according to Healey (1969), Wells and Awa (1970a), Watson and Martin (1972), and Nath (1972). In addition the spermatozoal flagellum, the main organ for motility was also found to be damaged by freezing (Koehler, 1966; Leverage, Valerio, Schultz, Kingsbury and Dorey, 1972; Pedersen, 1970 & 1972; and Nath, 1972).

The spermatozoal morphological changes throughout the different stages of the freezing process, i.e. dilution, equilibration and freezing followed by thawing after 24 hours and one month's storage in liquid nitrogen have not yet been traced, however.

The aim of the present work was to carry out an initial laboratory study of ram spermatozoa throughout the deep freezing process in order to detect and if possible prevent, the changes responsible for poor fertility of frozen ram semen.

In addition the characteristics, including spermatozoal morphology of the raw semen throughout the year were studied, with a particular view to the assessment of possible seasonal variation.

V REVIEW OF LITERATURE

1. SEMEN COLLECTION

Many authors have found that semen samples collected by means of the electro-ejaculator have an inferior quality and superior quantity to those obtained by the artificial vagina, in rams as found by Brady and Gildow (1939); Keast and Morley (1949); Blackshaw (1954b); Mattner and Voglmayr (1962); Salamon and Morrant (1963); Hulet, Foote and Blackwell (1964); Fraser (1968); Quinn, Salamon and White (1968a); Terrill (1969) and Visser (1969),in goats, Wilkins (1963) and Austin, Leidy, Krise and Hupp (1968), in bulls, Colleary and Ehlers (1964), and in both rams and bulls, Quinn and White (1966a) and Foote and Trimberger (1969).

Fraser (1968); Quinn et al. (1968a); Visser (1969) and Entwistle and Martin (1972), found that freezing results were poorer when ram semen, as well as bull semen (Colleary and Ehlers, 1964) was obtained by electro-ejaculation due to the larger quantities of the accessory fluids.

2. FACTORS EFFECTING SEMEN PRODUCTION

A) Volume

The two main parts of semen as ejaculated are the spermatozoa and the seminal plasma. The seminal plasma is a mixture of the accessory secretions and acts as a fluid medium for the spermatozoa and a source of nutrients, supporting sperm motility and metabolism (Mann, 1969). A very wide range of variability in the composition of the seminal plasma has been found by many investigators. It differs not only between species, but also within individuals as well as between different ejaculates

of the same individual (Mann, 1969).

The volume of the ram ejaculates usually ranges between 0.2 -3ml. with average of 1ml. (Starke, 1949; Lunca, 1964; Milovanov, Truibkin, Chubenko, Tsvetkov, Erzin, and Meschankin, 1964; Terrill, 1968 & 1969; Foote, 1969; Foote and Trimberger, 1969 and Fraser, 1971a).

B) Frequency of Collection

The higher the number of collection, the lower the volume and the sperm concentration per ejaculate in the rams as well as in other species (Salamon, 1962 & 1964; Lunca, 1964; Salamon and Lightfoot, 1967 and Sharma, Tewari and Roy, 1968).

A significant difference in the characteristics of ejaculates exists both within and between rams (Brady and Gildow, 1939; Starke, 1949; and Sharma et al., 1968); bulls (Davis and Williams, 1939; Swanney, 1953 and Bishop and Hancock, 1955); goats (Wilkin, 1963; Lunca, 1964 and Terrill, 1968); and in rabbits (Beatty, 1957).

The higher the semen collection frequency in rams the higher the susceptibility of spermatozoa to cold shock (Salamon and Lightfoot, 1967) and the lower the survival rate after deep freezing (Salamon and Lightfoot, 1967 and Entwistle and Martin, 1972). Increase in acrosomal damage has been observed to be associated with sexual rest in the bull (Wells and Awa, 1970b; Wells, Wondfrash, Awa and Stephens, 1970 and Wells, Awa, Jay and Fancy, 1971) and in the rabbit by Bedford (1964a).

C) Season

The male of domestic sheep does not show a restricted breeding season, so common in the female, but seasonal variations, in semen production and characteristics are evident (Asdell, 1965).

Sexual activity of the ram tends to be highest in the fall and lowest in the winter (Terrill, 1969), and is markedly inferior in spring and summer (Brady and Gildow, 1939; Koger, 1951 and Mohri, Hasegawa and Masaki, 1970). These changes are due to the extreme variations in the ambient temperature and day light length (Starke, 1949; Fowler, 1962; Bassett, 1963 and Terrill, 1969).

Ram semen collected during the non-breeding season shows poor quality, that is highly susceptible to cold shock (Misra and Sengupta, 1965) and deterioration in spermatozoal morphology (Starke, 1949; Juma and Dessauky, 1969 and Fraser, 1971a).

D) Light and Temperature

(i) In vitro

Incubation of the semen at a temperature above 37°C leads to a general disorganization of the entire sperm structure as observed by Saake and Almquist (1963b). White (1969) claimed that the light which normally prevails in the laboratory depressed the spermatozoal motility and the metabolic activity, and this condition is termed "photo-immobilization" by Mann (1964). Wales and Choong (1963) found that ultraviolet light depressed ram and bull spermatozoa especially after dilution with milk.

(ii) In vivo

The circulation of ice water (Chang, 1943) or the application of heat as well as the presence of thick wool around the scrotum of the ram (Moule and Waites, 1963; Rathore, 1969 & 1970; Smith, 1971 and Williamson, 1974a & b), led to lower semen quality especially by increasing acrosomal damage.

Similar effects have been reported in the bull (Lagerlof, 1934 & 1936) and the boar (Bane and Nicander, 1966).

3. SEMEN EVALUATION

A) Density

The density of semen, i.e. the number of sperms per unit of volume, is frequently required for semen evaluation. It can be determined by counting cells in a haemocytometer (Walton, 1927 and Brady and Gildow, 1939), but this method is very time consuming. The more rapid ways are based on the measurement of light transmission through spermatozoal suspensions, by means of a photoelectric colorimeter (Comstock and Green, 1939) or photometer (Willett and Buckner, 1951), or electronic method (Glover and Phipps, 1962 and O'Donnel, 1969). Recently Kihlstrom, Carlsson and Larsson (1975) introduced the cellscope automatic counting of spermatozoa in rabbit.

The spermatozoal concentration in the normal ram ejaculate, usually ranged between 2-5 x 10^9/ml. as cited by Starke (1949); Nalbandov (1964); Lunca (1964); Terrill (1968); Foote (1969) and Foote and Trimberger (1969). Good ram semen has a creamy appearance and consistency. Semen containing a high concentration, but mostly dead spermatozoa, has a brownish yellow appearance. As the concentration of the spermatozoa decreases, the semen becomes thinner and milky or watery in appearance (Terrill, 1968).

B) Motility

Motility is one of the most widely used tests for semen quality, in the international field of the artificial insemination. The proportion of progressively motile spermatozoa, rate of movement, and gross

abnormalities of the semen samples can be seen microscopically following dilution with physiological saline solution (Emmens, 1947 & 1948) with a thermostage at 38°C (Eane, 1952).

The "wave motion" created by spermatozoa swimming is a characteristic feature of the undiluted semen of rams (Rothschild, 1948 and Terrill, 1968) and bulls (Terrill, 1969). The spermatozoal speed in diluted semen sample varies with medium and temperature of storage (Bishop, 1962).

The simple microscopical evaluation procedures of the spermatozoal motility varies greatly. Alternatively, the semen may be allotted wave motion scores from 0-3 (Blom, 1946) or 0-4 (Emmens, 1947; Cragle, Myers, Waugh, Hunter and Anderson, 1955 and Wilkins, 1963) or 0-5 (Brady and Gildow, 1939; Lardy and Phillips, 1939; Starke, 1949 and Milovanov et al., 1964). The use of a photocell to measure the spermatozoal wave was reported by Glover (1968).

The individual motility may be graded, with steps of 5% or 10%, from 0 to 100% (Blom, 1946; Starke, 1949; Blokhuis, 1962; Emmens and Robinson, 1962; Mattner and Voglmayr, 1962; Mukhergee, 1964 and Milovanov et al., 1964). It is generally accepted that the motility of the normal undiluted ram semen ranges between 75-90% with good wavy appearance as cited by Starke (1949); Hulet and Ercanbrack (1962); Lunca (1964); Terrill (1968); Foote and Trimberger (1969) and Foote (1969)

C) Differential count

Studies of the spermatozoal morphology and the differential count between the live and dead cells on the basis of permeability of the cytoplasmic membrane of the dead spermatozoa to the vital stains were

reviewed by Hackett and Macpherson (1965a & b) and Fraser (1971b).

The vital stain of eosin or congo red was used in combination with suitable background stain such as opal blue (Lasley, Easley and McKenzie, 1942 and Easley, Mayer and Bogart, 1942), or fast green FCF (Mayer, Squiers, Bogart and Oloufa, 1951) or nigrosin (Blom, 1950). Thereafter the eosin nigrosin stain became universal especially after its modification by Hancock (1951), Swanson and Bearden (1951) and Hancock and Shaw (1955).

A fluorescence staining technique for the differential count of live and dead spermatozoa by tetracycline HCL stain as well as the morphology of the acrosome and the cell membrane has been introduced by Ericsson and Buthala (1970).

Emmens (1947) and Blackshaw (1958a) found that congo red was superior to eosin in staining dead spermatozoa for differential counts but Entwistle (1972) found no difference between congo red - fast green and nigrosin - eosin stains.

Swanson and Bearden (1951) found that the most suitable concentration of dyes were 1% eosin B and 5% nigrosin, but wide variation in their proportion did not alter the accuracy of the differential count. Dott and Foster (1972) found that the stained spermatozoa were less in 0.67% eosin and 10% nigrosin than in 0.67% eosin and 5% nigrosin.

Mayer et al. (1951) and Shelby and Foley (1964) claimed that careful control of stain concentration, pH, temperature and length of staining time are necessary, to minimize the effect of these variables on the staining ability.

Beatty (1957) and Buttle, Hancock and Purser (1965) found that some spermatozoa in nigrosin eosin preparations, with structurally

intact acrosomes also showed some evidence of staining with eosin. They were classified as living spermatozoa in spite of their altered staining property (Hancock, 1952). Fraser (1973) suggests that any spermatozoa stained completely with eosin be counted as dead.

For a satisfactory differential count, it would be advisable to avoid excessive dilution or inclusion of a protective agent such as egg yolk as found by Campbell, Hancock and Rothschild (1953); Bruce (1953); Jones and Martin (1965) and White (1969). In addition Jones and Martin (1965) found that the score of the stained spermatozoa increased with dilution of ram semen 10 or 40 fold and as the concentration of the sodium citrate increase in the diluent.

Similarly on inclusion of glycerol in the diluent, especially over 4%, the percentage of live spermatozoa decreased in a very disproportionate manner to the percentage of motile spermatozoa and predicted percentages of live spermatozoa, becoming extreme at the higher levels of glycerol (Bruce, 1953; Rowson, 1953; Mixner and Saroff, 1954; Cragle et al., 1955; Blackshaw, 1958a; Martin and Emmens, 1958; Martin, 1963a, d & e, & 1965a; Rathore, 1965; Jones and Martin, 1965; Jones, 1965b & 1969b and Turvey and O'Hagan, 1972).

The percentage of live spermatozoa or of abnormalities varies with different subjects (Branton and Salisbury, 1947 and Campbell et al., 1953), and with different staining and preparation techniques (Salisbury, Willett and Seligman, 1942; Wales, 1959; Buttle et al., 1965; Wilson, Warnick and Gutlerez, 1969 and Ball, Pickett and Gebauer, 1971).

The majority of investigators agree that the reaction time between the spermatozoa and the stain should be one minute. On the

other hand Rowson (1953) and Dott and Foster (1972), advised leaving the mixture longer, in order to get clear differentiation between the stained and unstained spermatozoa when egg yolk or glycerol was present in the diluent. Mixner and Saroff (1954); Eliasson and Treichl (1971) and Fraser (1973) agree that the stain does not give an exact assessment of the live percentage. The percentage of motile spermatozoa is judged to be the better criterion for spermatozoal viability especially when glycerol is present in the diluent.

Valid differential counts could be made between different semen samples, when both the method of preparing the smears and the criteria of assigning spermatozoa to the stained or unstained category are rigidly applied as stated by Dott and Foster (1972).

Campbell, Dott and Glover (1957) claimed that the thickness of the stain on the slide did not affect the proportion of stained spermatozoa. Because the differential count stains are not suitable for detailed acrosomal morphology, new staining techniques have been devised by Wells and Awa (1970a) and Turvey and O'Hagan (1972).

4. SPERMATOZOAL MORPHOLOGY

A) Normal

The spermatozoa represent only the final step in a series of complex changes (spermatogensis) in the testes, that govern their number and properties (Ortavant, Courot and Hochereau, 1969, and Mann, 1969).

The spermatozoa is a highly specialized condensed cell, which does not grow or divide. It is well known that in the majority of species the mature spermatozoa has a filiform structure and it is usually possible to distinguish three regions, head, neck, and tail. The

tail is divided into midpiece, mainpiece, and endpiece and the whole cell is surrounded by a cytoplasmic membrane (Figure 57).

i) The head of the spermatozoa

Nucleus

Mammalian sperm heads all have certain structural features in common but their shape is highly variable between different species (Fawcett, 1958).

It is a flattened ovoid structure in the ram (Randall and Friedlander, 1950, and Clermont and Leblond, 1955), in the bull (Hancock, 1952; Rahlmann, 1961; Kojima, 1962, and Saake and Almquist, 1963a, 1964a), and the rabbit (Bedford, 1964a & 1965b). Fawcett (1958) observed that in man the sperm head was oval when viewed on the flat and pyriform on edge. The head is V-shaped in the marsupials and in many rodents the head is shaped like a pruning hook (Fawcett, 1958; Phillips, 1972, and Jones, 1974).

The constituents of the spermatozoal head are, the plasma membrane, the outer and inner acrosomal membranes, the acrosomal contents, the nuclear envelope, and the nucleus containing condensed chromatin (Jones, 1971b and Friend and Fawcett, 1974).

In addition the spermatozoa of some rodents possess a slender refractile process known as the rod or perforatorium (Fawcett, 1958). It is generally observed that the nucleus occupies nearly the whole head of the spermatozoa. Its dense chromatin material is thickest near the junction of the midpiece, and gradually tapers anteriorly (Rahlmann, 1961). This dense material is composed mainly of deoxy ribonucleo-protein (Randall and Friedlander, 1950; Fawcett, 1958;

Saake and Almquist, 1963a, and Lake, 1966), which is the paternal hereditary material (White, 1958 & 1969; Ortavant et al., 1969, and Mann, 1969). The deoxyribonucleac acid (DNA) content is less in abnormal than normal spermatozoa but it does not differ between spermatozoa obtained from different regions of the male reproductive tract or different bulls (Gledhill, 1966a, b & c). In the human as well as the bull (Saake and Almquist, 1964a), stallion (Fawcett, 1958), sheep (Nath, 1972) and boar (Andersen, 1974), the spermatozoal nucleus has vacuoles which are not important organelles and it has been suggested that these vacuoles are to be considered within the limits of normal structure. The spermatozoal head is enclosed by a membrane, which appears to be continuous with the outer membrane of the midpiece and tail. Particularly in the head region the membrane appears to be double walled (Fawcett, 1958).

Acrosome

The acrosome, a cap like structure which covers the anterior surface of the spermatozoal head, has been found to occur in the spermatozoa of all mammals so far studied by the investigators since Lenhorsok (1898) (after Fawcett, 1958) who applied the term "Head or acrosome cap". Andersen, (1974) cited that the acrosome is of vital functional importance in the mechanism of fertilization and any defects associated in this structure led to the improper fertility of the spermatozoa.

It is generally accepted that the acrosome consists of an outer and inner membrane, enclosing the homogenous electron dense materials or the opical body (Bedford, 1964a & b, and Jones, 1971b & c). It is a bilaminar structure as seen in spermatozoa of the rabbit (Bedford, 1964a and 1965b), the bull (Kojima, 1962) and the boar (Jones, 1973a).

Kojima (1962) claimed that the outer and inner acrosomal membrane each has a double layer.

Sagittal and transverse sections of the spermatozoal head show that the relative proportions of the acrosome cap vary in different regions. The anterior and antero-lateral regions are comparatively much thicker than that of the posterior (Bedford, 1964a & b; Bane and Nicander, 1966; Jones, 1971c, and Andersen, 1974). It is apparent that the plasma membrane is always in close apposition to the external surface of the post nuclear cap, and over the anterior and lateral edges of the acrosome (Jones, 1973a).

The posterior acrosomal part usually overlaps the post nuclear cap to form the equatorial segment as has been found in the spermatozoa of the ram (Jones, 1973a & c), rabbit (Bedford, 1964a), the boar (Hancock, 1957; Nicander and Bane, 1962, and Jones, 1971c) and the bull (Rahlmann, 1961). The surface of the acrosome is smooth in all domestic animals as found by all workers except Lung and Bahr (1972) who claimed that a droplet like elevation was present in human spermatozoa. H yaluronidase enzyme which is necessary for the ovum penetration and fertilization is present in the acrosomal materials (Rao and Berry, 1949; Hancock, 1962; Penn and Gledhill, 1972; Gaddum-Ross and Blandau, 1972, and Talbot and Franklin, 1974).

The acrosome covers nearly two-thirds of the nucleus in the ram (Clermont and Leblond, 1955), the boar (Hancock, 1957, and Bane and Nicander, 1965, 1966), the rabbit (Bedford, 1964a), and bull (Lewis and Dickey, 1968), or little less (half) in the bull as found by Hancock and Trevan (1957); Kojima (1962); Saake and Almquist (1964a) and Bernstein and Teichman (1972), while in guinea pig spermatozoa the acrosome nearly approaches the size of the nucleus (Fawcett, 1958).

The subacrosomal space which is present between the inner acrosomal membrane and the nuclear membrane has been observed by all investigators as existing in the spermatozoa of all domestic animals but not of man (Bedford, 1967). Bedford (1964b & 1965b) observed a significant difference in the acrosomal measurements in different stages of the spermatozoal maturation in rabbit as did Fawcett and Hollenberg (1963) in the guinea pig. Those obtained from the caput with proximal cytoplasmic droplets were larger than those obtained from the cauda epididymis with or without distal droplets. It would appear that a marked reduction in acrosome size and release of the droplets occurs by the time of spermatozoa approaching the cauda, when they became functionally mature. Branton and Salisbury (1947) found no difference in morphology of spermatozoa from different parts of the genital tract of bulls but did find differences between bulls.

Post-nuclear cap

The post-nuclear cap surrounds the base of the nucleus and is covered by the cytoplasmic membrane of the cell (Hancock, 1957; Hancock and Trevan, 1957; Saake and Almquist 1963a & 1964a; Wu, 1965; Wu and Newstead, 1966; Wooley, 1970; Wooding and O'Donnell, 1971, and Bernstein and Teichman, 1972). Fawcett (1958) claimed, however, that the post-nuclear cap does not exist. It is formed of fibrous proteins rich in sulpher (Ortavan et al., 1969) and it is easily impregnated with stains containing silver (Hancock, 1957, and Hancock and Trevan, 1957) and eosin (Hancock, 1952; Beatty, 1957; Rahlmann, 1961; Saake and Almquist, 1964a; Buttle et al., 1965; Dott and Foster, 1972, and Fraser, 1973), in spite of the anterior part of the cell remaining clear.

This phenomenon is due to the presence of holes or perforations in the post-nuclear cap of bull spermatozoa (Rahlmann, 1961; Saake and Almquist, 1964a; Veres and Ocsenyi, 1968b; Koehler, 1970, and Wooding and O'Donnell, 1971), and of human sperm (Koehler, 1972). Friend and Fawcett (1974) found that the posterior portion of the nuclear envelope of the rat as well as the guinea pig spermatozoa (Koehler, 1973) showed nuclear pores and corrugation of the plasma membrane after freezing. Wu and McKenzie (1955) found that ram spermatozoa shows the appearance of granules and holes in most portions of the head. Nath (1972) found that at the posterior end of the ram spermatozoa the nuclear envelope is not firmly attached to the chromatin, and appears to be bulging. Saake and Almquist (1964a) observed the presence of a porous double layered membrane covering the nucleus just under the post nuclear region in bull spermatozoa. The post nuclear cap is firmly attached to the base of the nucleus, but became progressively looser anteriorly.

Dott (1969) found that the pores were present in the posterior border of the equatorial region and loosening of the covering membrane in the posterior region of the bull, ram and rabbit spermatozoa. Bedford (1963a, 1964a & b, and 1965a), Koehler (1966), Hafez and Kanagawa (1973) and Flechon (1974) observed a narrow line with dotted impressions and irregular appearance in the posterior third of the rabbit spermatozoal head.

ii) Neck

The sperm neck is the implantation region of tail. It consists of the proximal centriole and the implantation plates (Kojima, 1962; Wu and Newstead, 1963, and Saake and Almquist, 1964a).

The implantation region appears, as a cylindrical structure, composed of two major columns fused at their anterior end to form an articular structure in close apposition to the basal nuclear indentation (Wu and Newstead, 1966) or partially fused to the nuclear envelope (Friend and Fawcett, 1974) with a double membrane system, the basal plate and the lamellar character of the implantation zone (Veres and Ocsenyi, 1968b).

In boar spermatozoa the neck consists of a number of fibres gathered into three granules which lie at the base of the head and appear to be related to the insertion of the neck fibres (Hancock, 1957).

iii) Tail

The tail provides a means of locomotion. It propels the spermatozoa by waves which are generated in the implantation region and pass distally along with the tail like a whiplash. It differentiates into three parts, mid, main and end pieces. The anterior end of the midpiece connects with the head at the implantation region or fossa (Blom and Andersen, 1960).

The spermatozoal tail of the domestic animals has an axial core containing 2 central fibrils surrounded by concentric rings of 9 inner and 9 outer fibrils. Each of the 9 fibrils appear to have even finer subunits in man, bull, ram, boar and rabbit spermatozoa (Wu and McKenzie, 1955). The 2 central fibrils are connected by fine threads while fine radiating spokes connect them with 9 inner fibrils (Wu and Newstead, 1963 & 1966). The outer coarse fibres are clearly in continuity with the connecting segment (implantation region) in the ram (Wu and McKenzie, 1954; Ortavant et al., 1969, and Nath, 1972), and in the bull (Blom and Andersen, 1960, and Saake and Almquist, 1963a & 1964b), as well as in the boar (Hancock, 1957).

Midpiece

The thickest region of the tail is the midpiece which is regarded as an important powerhouse for supplying energy, and carrying the enzymes which are necessary for motility, respiration and metabolism (Mann, 1969).

In the midpiece at least 3, possibly 4, of the outer 9 fibrils are larger than the others (Wu and McKenzie, 1955; Blom and Andersen, 1960, and Rahlmann, 1961). The fibres decrease in thickness gradually along their course posteriorly along the tail (Wu and Newstead, 1966).

The mitochondria are double walled and possess internal cristae that are vesicular or tubular in structure (Saake and Almquist, 1964b). The mitochondrial sheath is divided into the pars spiralis and the pars ascendens. The pars spiralis is made up of about 75 (Rahlmann, 1961) to 100 (Blom and Andersen, 1960) individual turns helically coiled around the axial filament and they are quite uniform in sheep spermatozoa (Nath, 1972).

The cortical helix is composed of numerous granules or a series of dark bands (Rahlmann, 1961) lying together within the clearly visible tubular mitochondria. The fibrils of the axial filament and the helical structure have a continuation from the neck to the tail end of the spermatozoal cell (Wu and McKenzie, 1955, and Wu and Newstead, 1963).

The proximal and distal centrioles are located at the upper and lower ends of the midpiece respectively (Blom and Andersen, 1960, and Hancock, 1962). The centriolar wall consists of 9 sets of triplet filamentswhich appear circular in cross section (Wu and Newstead, 1963 & 1966).

In the anterior part of the epididymis, the neck of most spermatozoa has a cytoplasmic droplet, which migrates towards the distal end

of the midpiece, from which it is later eliminated as a rule of maturation (Lasley and Bogart, 1944; Branton and Salisbury, 1947; Rao and Berry, 1949; Hancock, 1953, 1955 & 1957; Bedford, 1965b; Wu and Newstead, 1966, and Dott, 1969). The normal cytoplasmic droplet differs completely from the pseudodroplet (Blom and Andersen, 1968). The droplet appears as a membrane structure and consists of a series of concentric shells or rings of electron dense elements (Wu, 1965 & 1968) or endoplasmic reticulum (Wu and Newstead, 1963). The tubular and vesicular contents of the droplet are usually reduced during its passage along the midpiece (Bedford, 1965b). Alkaline phosphate activity is present in the droplets of the ram and rabbit spermatozoa (Moniem and Glover, 1972).

Mainpiece

The mainpiece is the longest part of the tail and provides most of the propelling mechanism. The 9 coarse fibres of the outer ring diminish in thickness and finally disappear leaving only the central 2 and 9 inner fibrils in the axial core for most of the length of the mainpiece (Saake and Almquist, 1964b, and Wu and Newstead, 1963 & 1966). Such changes in the axial core start in the midpiece in the fowl spermatozoa (Lake, Smith and Young, 1968).

Endpiece

The endpiece is the short terminal portion of the tail. It contains only the axial bundles and it is neither surrounded by a sheath nor the outer 9 fibres (Kojima, 1962; Saake and Almquist, 1964b; Nalbandov, 1964; Wu and Newstead, 1966, and Lake et al., 1968).

B) Abnormal

i) Under the light microscope

Acrosome abnormalities

Acrosomal abnormalities are classified as hereditary (Hancock, 1949 & 1953) and secondary or environmental, such as after sexual rest of the bull (Blom, 1945; Wells and Awa, 1970b, and Wells et al., 1970 & 1971), or after dilution, storage at 5°C and freezing of the bull spermatozoa (Wells and Awa, 1970a) as well as of the ram spermatozoa (Watson, 1975b). Acrosomal damage usually takes place in a series of progressive changes in the acrosomal membranes and their contents. Such changes were termed as a non-specific acrosomal reaction (Bedford, 1968 & 1969) and classified according to Wells and Awa (1970a) in the following forms:

1) Normal acrosome was defined as being closely adherent to the sperm cell head, smooth and entire in shape.

2) Typical abnormal conditions observed included

a) swelling or thickening, especially at the anterior tip of the spermatozoal cell,

b) irregularities in the shape of the acrosome,

c) disintegrating acrosome,

d) elevated and knobbed,

e) combined conditions of c and d.

General spermatozoal abnormalities

Generally the frequent spermatozoal abnormalities as found in the semen of the domestic animals include the following:

a) Free heads

Free heads are usually common in the semen of sterile or infertile males (Lagerlof, 1934; Hancock and Rollinson, 1949; Williams, 1965; Settergren and Nicander, 1968; Fraser and Penman, 1971, and Williamson, 1974b). Settergren and Nicander (1968) claimed that most of the free heads in bull semen exhibit a dark acrosomal margin without normal smooth outline.

b) Pyriform heads

Spermatozoal heads with pear or pyriform shape are usually smaller than normal. The nuclear portion of the head is usually reduced in its transverse diameter. Not infrequently the longitudinal diameter is similarly affected with tapering towards the base (Starke, 1949, and Coubrough and Barker, 1964).

c) Narrow heads

Spermatozoa with entire head reduced in its transverse diameter. There is no distinct tapering towards the base as in pyriform heads.

d) Round heads

e) Giant heads

f) Dwarf heads

They are small heads less than half the normal in diameter. The posterior end of some dwarf spermatozoal heads were constricted, with pyriform appearance. Such abnormality is usually associated with disturbed spermatogenesis in rams (Starke, 1949) and bulls (Lagerlof, 1934, and Salisbury and Vandemark, 1961a).

g) Double heads

Veres and Ocsenyi (1968a) claimed that double heads are due to the accidental adhesion or incomplete division (Starke, 1949) of

the spermatid nuclei in the testes. In addition triple and quadra heads might be seen on occasions in rams (Starke, 1949), bulls (Salisbury and Vandemark, 1961a), or boars (Kojima, 1973).

h) Broken neck

Complete broken neck or just bending of the head over the midpiece has been seen in rams (Fraser and Penman, 1971).

i) Broken, short or long midpiece

j) Presence of the cytoplasmic droplets

The usual sites of the cytoplasmic droplets were either at the proximal or the distal end of the midpiece. The pseudocytoplasmic droplets are usually situated near the centre of the midpiece length (Blom, 1968, and Blom and Andersen, 1968). The distal end of the midpiece appears to be particularly susceptible to distortion and often it is bent round a droplet (Starke, 1949; Dott, 1969; Pedersen and Lebech, 1971, and Pedersen, 1972).

The presence of the droplets in a proximal position signifies immaturity or a defect in the maturation process of the spermatozoa (Lagerlof, 1934 & 1936; Rao and Berry, 1949; Salisbury and Vandemark, 1961a, and Dott, 1969).

k) Filiform midpiece

The filiform abnormality of the midpiece is characterized by the absence of the cytoplasmic membrane leaving the axial filaments core denuded (Starke, 1949).

Fragmentation and disorganization of the midpiece as well as partial or complete loss of the mitochondria sheath have been observed in bulls (Salisbury and Vandemark, 1961a; Coubrough and Barker, 1964) and in stallion (Chenoweth, Pascoe, McDougall and McCosker, 1970).

l) Enlarged midpiece

A spherical swelling usually appears just below the neck, but sometimes the swelling involves the whole length of the midpiece. Kojima (1973) claimed that a thick flagellum arose from fusion of several flagella.

m) Double midpiece or tail

Starke (1949) suggested that double midpiece or flagellum happens in the same way as the double head which might be due to an incomplete division of the spermatid nuclei in the testis.

n) Abaxial attachment of the midpiece

Sometimes the tail attachment is slightly shifted to one side, and in extreme cases it is right on the edge of the spermatozoal head (Starke, 1949; Salisbury and Vandemark, 1961a, and Settergren and Nicander, 1968).

o) Coiled tails

Various types and degrees of coiling have been observed in the spermatozoa of different species. Sometimes the tail enveloped the head closely, in other cases it was coiled at the base of the head and more frequently it looped back on itself near the end of the midpiece (Starke, 1949; Pedersen and Lebech, 1971, and Pedersen, 1972).

In addition there is a simple coiling (Crooks) as described by Fraser (1973), and sometimes the tail coiled around its terminal part (Starke, 1949).

Blom (1959 & 1968) observed a zigzag or corkscrew shape of the tail coiling, especially involving the midpiece of the bull spermatozoa. Such conditions were usually associated with disturbances in the arrangement of their fibres. Quinn et al., (1968b) and Quinn, White and

Cleland (1969), observed that bull and ram spermatozoa exhibit coiling or spiralization, as well as swollen tails (Drevius and Eriksson, 1966, and Drevius, 1972) after dilution and preservation in hypertonic solutions.

Drevius (1963) claimed that the hypertonic solutions of glycerol evoked the curving and bending of the spermatozoa tails and effect their fibrilar systems. Bostrom and Rubin (1973) found a high percentage of tail coiling following cold shock as well as freezing of human spermatozoa.

ii) Under the electron microscope (E.M.)

Variations in the size and shape of the spermatozoa are the results of abnormalities of different anatomical components of the spermatozoa. A single anatomic component such as the nucleus, acrosome, the mitochondrial sheath or the cytoplasmic membrane of the spermatozoa, provides most of the abnormalities as seen with the electron microscope and reduces the fertilizing capacity of the sire (Williams, 1964).

Bane and Nicander (1966) found that the thickness of the posterior part of the acrosome in boar spermatozoa led to absence of the equatorial segment and an apparent abnormal shape of the nucleus, but when the thickness concentrated in the anterior part of the acrosome of the boar spermatozoa, the acrosome took the shape of a tongue-like protrusion.

Bane and Nicander (1965), found that the invagination of the nuclear membrane into the acrosome making a pouch, in bull and boar spermatozoa as a prematurity abnormality.

Swollen acrosomes with irregular staining intensity (Saake and Amann, 1966, and Veres and Ocsenyi, 1968a) as well as inward inversion (Aamdal, 1951) or irregular acrosomal margin (Settergren and Nicander, 1968) have been observed in the ejaculated bull semen. Hancock and Trevan (1957), Veres (1964), Coubrough and Barker (1964) and Veres and Ocsenyi (1968a), have seen partially missing or cut acrosomal edges in bull spermatozoa. Schroder (1964) found that in the ejaculated bull semen, the number of morphological changes was higher in living than in dead spermatozoa and highest during winter time. Singh and Sadhu (1966) found a decrease in the spermatozoal head dimensions of the bull and buffalo spermatozoa on storage at $5^{o}C$. On the contrary, Blackshaw and Salisbury (1972) found no significant change in the sperm size and Baicoianu and Dimoftache (1968) found a slight increase in the spermatozoal head measurements after storage of ram semen at $4^{o}C$ for 5 days but no change in the bull spermatozoa. Mukherjee (1964) observed an increase in thickness of the head, midpiece and tail due to loss of phosphalipids after storage or cold shock.

Phillips (1972) found that the plasma membrane which cover the acrosome of the hamster and mouse spermatozoa contains minute vesicles and tubules in specific regions which seem to have a "sticky" quality. In living spermatozoa, this area of the spermatozoalhead was observed to stick to other spermatozoal heads or to the substrate.

Bedford (1965c & 1970) suggested that head to head or tail to tail agglutination in rabbit, ram, and bull spermatozoa were due to the presence of specific antibodies in the media.

Lindahl and Kihistrom (1954) and Lindahl (1968a & b, & 1973) found that some cat-ion activates the ATP (adenosine triphosphate) which

is present in the membrane covering the acrosome of the bull spermatozoa and leads to sperm head agglutination.

Mammalian spermatozoa subjected to unfavourable environment, such as dilution, storage in vitro at room temperature or after cooling to low temperature on freezing stress, present a lot of morphological changes. The acrosomal changes usually take place in a series of non-specific degeneration or reaction (Bedford, 1968 & 1969) and are described by Williamsons (1974b) in ram, Saake and Almquist (1963b) and Saake and Marshall (1968) in bull, and Jones (1973a & c) in boars. They include swelling, vesiculation and disintegration of the acrosome. Initially the cell membrane fragments, the anterior acrosome swells and a line of vacuoles appears within its marginal thickening. The anterior acrosome and then the equatorial segment of the acrosome disintegrates, leaving the nucleus surrounded anteriorly, only by the inner acrosomal and nuclear membranes and posteriorly by the post nuclear sheath and finally complete acrosomal loss might take place.

Nath (1972) found that the ram spermatozoal acrosome was the actual site of freezing damage. In addition the degenerative changes involve the midpiece as well as the mainpiece leading to considerable distortion of the midpiece fibrils and the mitochondrial cristae. In 40-50% the fibrils aggregated along one side of the midpiece leaving a cavity on the outer side as an ice cavity.

Pedersen (1972) found that the midpiece matrix became thinner in human spermatozoa after freezing.

Koehler (1966) and Leverage et al., (1972) found that damage includes the breakage of mainpiece plasma membrane of the bull spermatozoa. The fibers are much less distinct with coiled endpiece along the distal

end of the mainpieces, both being enclosed in a common cell membrane (Pedersen and Lebech, 1971, and Pedersen, 1972).

According to the treatment severity, partial or complete degeneration has been observed in the spermatozoa after cooling to low temperature, cold shock or storage in vitro of the boar spermatozoa (Bane, 1961; Boender, 1968; Pursel, Johnson and Rampacek, 1970 & 1972, and Jones, 1972a), of the ram (Walton, 1957; Hill, Godley and Hurst, 1959; Quinn, et al., 1968a & 1969; Graham, Graves, Sharma and Salisbury, 1971; Jones and Martin, 1973, and Srivastava, Munnell, Yang and Foley, 1974), and of the bull (Hancock, 1952; Blom, 1964; Zvereva, 1964; Koehler, 1966; Quinn et al., 1968b; Dott, 1969; Ericsson and Buthala, 1970, and Jones, 1972b). Varnavaskii (1970a & b), Visser and Salamon (1974) and Butler and Roberts (1975) found that the severity of the morphological damage depended on the diluent composition. They were higher in the absence of either egg yolk, certain phospholipids or glycerol.

The severity of the changes on the spermatozoal morphology, especially the acrosome, was greater after freezing than after cooling, cold shock or storage, in ram (Quinn et al., 1968a & 1969; Healey, 1969; Nath, 1972; Watson and Martin, 1972, 1973a & b & 1974, and Watson, 1975b), in bull (Lovelock and Polge, 1954; Saake and Almquist, 1961; Veres and Ocsenyi, 1968b; Wells and Awa, 1970a, and Leverage et al., 1972), in man (Pedersen and Lebech, 1971; Koehler, 1972; Pedersen, 1972; Leverage et al., 1972, and Friberg and Gemzell, 1973), in rabbit (Smith and Polge, 1950a; Lovelock and Polge, 1954; Bedford, 1964a & 1965a; Koehler, 1970, and Flechon, 1974), in fowl (Harris, Thurston and Cundall, 1973), and in rat (Friend, 1973). Layet and Hodapp (1938) found that

frog spermatozoa became swollen with disintegration and losing of refringence after freezing. Watson and Martin (1974) found that the great change in the ram spermatozoa occurred between -5°C and -15°C during their freezing, which might be due to the intracellular salt concentration and ice crystalization. There is a great release of phospholipids from the spermatozoa of different species after cold shock (Mann and Lutwak-Mann, 1955; Walton, 1957; Blackshaw and Salisbury, 1957; Wales and White, 1959; Quinn et al., 1968b; Pursel et al., 1972a, and Butler and Roberts, 1975), or after deep freezing (Graham and Pace, 1967; Platov, 1968; Nauk, Ljansberg and Seikin, 1970; Nauk and Skvorcova, 1970; Nath, 1972, and Coulter and Foote, 1975), or during both conditions (Quinn et al., 1969; Darin-Bennett, Poulos and White, 1973; Darin, Poulos and White, 1973; Polge and Butler, 1973, and Hibbitt and Moore, 1975). The release of the glycolytic enzymes by the bull, boar and ram spermatozoa after cold shock (Harrison and White, 1972), or just after cooling to 5°C of the boar spermatozoa (Hibbitt and Moore, 1975) and of the reduction in the DNA contents of ram spermatozoa after freezing (Quinn and White, 1968, and Darin-Bennett et al., 1973), have been found due to the plasma membrane damage after such treatments.

The morphological damage after freezing in protective diluents was greater in boar and ram than in bull spermatozoa (Healey, 1969; Milovanov, Varnavaskii and Nauk, 1970; Varnavskii, 1970b; Watson and Martin, 1972, and Darin-Bennett et al., 1973).

Pursel, Johnson and Leisure (1970), Pursel et al., (1972a), Jones (1972b), Jones and Holt (1974) and Pursel, Johnson and Schulman (1974), found that the addition of the diluents itself led to the morphological

damage to the boar spermatozoa with reduced motility.

Healey and Weir (1970) found that the dilution of the Chinchilla spermatozoa with ringer solutions caused a detachment of the outer membrane of the tails and the disruption of the acrosome.

The cryoprotective agents such as the glycerol or dimethyl sulphoxide (DMSO) increase the morphological damage to the spermatozoa after incubation at 5 or 20°C (Smith and Polge, 1950a; Almquist, 1959; Marshall, Saake and White, 1968, and Jones, 1973a), and especially after freezing (Harris et al., 1973). The reduction in the concentration of such cryoprotective reduces the damage to some extent but doesnot prevent it (Smith and Polge, 1950a; Saake and Almquist, 1962, and Harris et al., 1973). The addition of egg yolk to the diluent reduces the degree of the degenerative changes of the ram spermatozoa after cooling as well as freezing (Watson and Martin, 1973a & b, and Jones and Martin, 1973).

Jones and Martin (1973) observed that the acrosomal and mitochondrial damage were higher in diluted ram semen incubated at 35°C than at 5°C.

Saake and Almquist (1963b) observed that the dilution of bull spermatozoa in hypertonic solutions led to the damaged or missing cell membrane.

Wiggin and Almquist (1975a) found that for each 20°C rise in the thawing temperature over the range of 35-95°C, intact acrosome increased a mean of 6.6 and 4.4% units while spermatozoal motility increased by 2.4 and 1.6% units at 0.5 and 2.0 hours of equilibration of the bull semen respectively. Varnavskii (1970b) found that morphological defects were higher after rapid freezing than slow freezing in boar semen. Robbins, Gerber and Saake (1972) found greater retention

of bull spermatozoal acrosome after thawing at higher temperature (35°C and 75°C) than at low temperature (5 and 20°C) but incubating thawed samples at 37°C or ageing (Saake and White, 1972) increased the acrosomal damage.

Garner, Graves, Sharma and Salisbury (1971) found that the specific phospholipids decreased and the acrosomal damage increased in ram spermatozoa after storage at 14°C or incubated at 37°C for four hours. The same findings have been observed in bull (Marshall and Saake, 1967), rabbit (Gulyas, 1966) and fowl spermatozoa (Lake, 1966). Morphological abnormalities and spermatozoal disintegration associated with lost acrosomes were observed to be frequent after application of mild heat around the testicle of the ram (Moule and Waites, 1963; Rathore and Yeates, 1967; Rathore, 1968, 1969 & 1970, and Williamson, 1974a & b), and bull (Lagerlaf, 1934) or ice water circulating around the testis of the ram, bull, boar, rat and rabbit (Chang, 1943).

Extensive acrosomal loss and plasma membrane damage with invagination of the two membranes occur in rabbit spermatozoa when recovered from the female uterus after insemination (Bedford, 1963b, 1964a & 1965a and Barros, Bedford, Franklin and Austin, 1967). Similar findings have been observed in the semen of the male rabbit (Bedford, 1963a, 1964b & 1965b) and of the bull (Davis and Williams, 1939; Wells and Awa 1970b and Wells et al., 1970 & 1971) following sexual rest. However, Wells et al., (1970) claimed that the integrity of the acrosome is highly subjected to environmental and physiological changes or other unknown stresses.

Ultrastructural changes have been observed in the untreated (control), fresh ejaculated spermatozoa (Eane, 1961) or physiological

disorders (Hafez and Kanagawa, 1973) or after pathological conditions (Hancock, 1953, and Bane, 1961) or as a result of artefact (Zamboni and Stefanini, 1968 and Jones, 1973b). Johnston and Reid (1972) and Jones (1972a) claimed that the buffer dilution of the spermatozoa before fixation, increases the susceptibility of the spermatozoa to morphological artefacts, especially the breakage of the plasma membranes. Jones (1972a & 1973c) found that acrosomal damages were highest in ram, less in boar and lowest in bull spermatozoa after low buffer dilution before fixation. Bedford (1964a) claimed that the appearance of extreme ballooned acrosomal membranes as free or loosely adherent, might be partially due to fixation artefacts.

Healey (1969) and Leverage et al., (1972) observed that where the criterion for successful preservation by freezing as well as the storage of the spermatozoa at low temperature (0-5°C) (Lagerlof, 1934 & 1936, and Bane, 1961) has been based on motility, this can be misleading with regard to fertility. Furthermore samples of spermatozoa containing high percentage of disintegrating cells may have acceptable motility, but this is not indicative of their true potential fertility.

C) Capacitation

Capacitation or the true acrosomal reaction, which is observed during the fertilization process is completely different from that of the non-specific degeneration (Bedford, 1969). The true acrosomal reaction includes the fusion and vesiculation of the plasma and outer acrosomal membrane with loss of the acrosomal contents while the non-specific reaction takes place as a result of senility or death of the spermatozoa due to the environmental stress (dilution, cooling, freezing,

etc.). The steps of the non-specific degeneration were characterized by swelling of the acrosome, vesiculation of the acrosome followed by rupture of the plasma and acrosomal membranes and loss of the acrosomal contents. The final step is the complete removal of the acrosome (Yanagimachi, 1969, and Austin, 1972 & 1975). Such reactions have been observed in rabbit by Moricard (1961), Bernstein (1966), Bedford (1968 & 1969) and Overstreet and Bedford (1974), of the rodents by Austin (1960, 1961 & 1964), Gwatkin and Andersen (1969), Yanagimachi (1969) and Hartmann and Gwatkin (1971).

Blom (1964) and Jones (1972a) claimed that the non-specific acrosomal degeneration followed the same procedure of the true acrosomal reaction, which takes place just before fertilization. Bedford (1963b) claimed that spermatozoa which have shed their acrosome prior to fertilization are those which have been fully capacitated, but it might be due to the senility state (Hancock, 1955 & 1957, and Bedford, 1963a).

D) Post mortem changes of the spermatozoa

The degenerative changes which usually occur after cessation of spermatozoal motility (Hancock, 1953) show an acrosomal and plasma membrane breakage, with leakage of their contents (Saake and Almquist, 1964a, and Saake and Marshall, 1968).

In general, dead or killed spermatozoa exhibit an absence or indistinct acrosomal border which is thick and well defined in the living one. There is an apparent loosening of the attachment of the outer acrosome and its posterior border became distorted. Disintegration of the outer acrosomal membrane of the dead spermatozoa may be followed by loss of the inner membrane as well as the post nuclear cap (Hancock, 1953).

The appearance of the equatorial region is more clear in the dead spermatozoa especially after loss of the acrosome (Hancock, 1952).

Emmens (1947) found that just after the death of rabbit spermatozoa the tails stained before the heads during staining with eosin nigrosin stain.

E) Correlation of spermatozoal viability and morphology

There was a positive correlation between the spermatozoal dead percentage and the incidence of the abnormal morphology of bull semen (Bishop, Campbell, Hancock and Walton, 1954, and Bishop and Hancock, 1955), of ram (Hulet, Warren, Foote and Blackwell, 1965) and of rabbit (Beatty, 1957).

Saake and Marshall (1968) observed a positive relationship between the soundness of the spermatozoal acrosome and the motility, i.e. immobile spermatozoa usually associated with greater acrosomal disintegration.

Cragle et al., (1955) found that the results obtained by live percentage count with vital stain were not consistent with average for visual estimation of the motility scores. On the contrary, Lasley (1951) observed a positive high significant correlation between motility and live percentages in both the fresh and stored semen.

Nour-Eldin, Omar, Younes and Abboud (1969) found that the correlation between motility and live percentage increased with low concentration of the vital stain and vice versa. Also they claimed that some spermatozoa might be still alive at the moment of staining, but were not motile (Eliasson and Treichl, 1971).

Lasley (1944), Bishop, et al., (1954) and Bishop and Hancock (1955), found a positive significant direct relationship between motility and live spermatozoal concentration as well as the incidence of dead spermatozoa and spermatozoal abnormalities.

Hancock (1951) found that the live percentage of the spermatozoa decreased as the difference between the temperature of semen and stain increased.

Hancock and Shaw (1955) found that any increase in the dead percentage lead to an increase in the incidence of spermatozoal agglutination.

Semen samples with high acrosomal abnormalities of their spermatozoa (Bane, 1961) as well as increasing the incidence of dead spermatozoa (Bishop and Hancock, 1955 and Beatty, 1957) lowered the resistance of the spermatozoa to cold shock. Hill et al., (1959), Martin (1963b) and Jones (1965b) found that there was a positive correlation between spermatozoal damage and decline in their motility with the length of the equilibration time as well as the storage in vitro (Campbell, 1953, and Martin, 1963b).

It is well known that the freezability of the semen usually differs among the sires as well as the ejaculates of the same bull (Swanney, 1953, and White, Blackshaw and Emmens, 1954), and ram (Salamon, 1968, and Sainsbury, 1968).

It has been generally accepted that the spermatozoal activity decreased and their morphological abnormality increased as the glycerol and other cryoprotectives, concentration increased in the diluents (Miller and Vandemark, 1953; Blackshaw, 1958a; Martin, 1963d; Jones, 1965b & 1971c, and Salamon, 1968).

Blackshaw (1953) cited that the spermatozoal motility was better in the samples with higher dilution rate than those with lower dilution rate.

Davis and Williams (1939) observed that the motility of the bull spermatozoa decreased as the pH of the diluent increased beyond the neutral. Page, Gebauer and Pickett (1968a) and others who prefer the cooling of the diluted semen before glycerolization found that the post-thawing motility became higher as the time is longer. Aamdal and Andersen (1968a & b), Andersen and Aamdal (1972) and Roussel, Abilay, Dragland, McNabb and Guthrie (1974) found a positive relationship between the thawing temperature and motility and acrosomal soundness.

Saake and White (1972) and Robbins et al. (1972) found that the spermatozoal motility decreased and their morphological abnormality increased as the post thawing incubation time at 37°C increased.

F) Significance of morphological abnormalities

In general normal fertile males (ram, bull or boar) produce around 90% of normal spermatozoa as stated by many authors.

On the other hand the male became less fertile if his semen exhibits more than 20% abnormal spermatozoa as found in bull (Lagerlof, 1936; Hancock, 1949, and Hancock and Rollinson, 1949) and in ram (Hulet and Ercanbrack, 1962).

Green and Comstock (1939) and Terrill (1968) claimed that spermatozoal head abnormalities tend to lower the ram fertility greater than those of the tail. However, Wu (1966) and Lindemann and Rikmenspoel (1972), claimed that more infertility was attributable to morphological defects of the tail (midpiece).

5. ULTRA LOW TEMPERATURE (DEEP FREEZING) PROCEDURE

A) Dilution

Spermatozoa do not survive for more than a matter of hours after collection in vitro (Willet, Fuller and Salisbury, 1940) because seminal plasma itself is harmful for the spermatozoa of some species, e.g. boar and stallion (Smith and Polge, 1950a &b) and there is an alteration in pH due to spermatozoal activity (Mann and White, 1957, and Mann, 1964) and bacterial growth (White, 1969). In addition rapid cooling of the semen to the storage temperature subjects the spermatozoa to a temperature shock (Easley et al., 1942; Lasley and Mayer, 1944, and Lasley and Bogart, 1944), which kills most of the spermatozoa and increases the rate of tail coiling. Even at low temperature preservation, harmful metabolic products such as lactic acid (Mann, 1964) accumulates at a relatively rapid rate and eventually reaches a concentration sufficiently great to kill most of the spermatozoa.

Most of the semen diluents in the world today are based on the milk or egg yolk or their combination to provide energy as well as protecting them against cold shock during cooling and freezing. The addition of sugars is necessary to provide the spermatozoa with nutrients and the proper tonicity of the diluent.

Buffers such as sodium citrate or phosphate, might be added for the pH adjustment of the spermatozoal environment.

To minimize the harmful effect of the freezing on the spermatozoa a cryoprotective agent such as glycerol or dimethyl sulphoxide (DMSO) has to be added to the ingredients of the diluent.

Sulphanilamide and antibiotics are used in diluents, especially in contaminated samples, for bacterial control.

pH

In general spermatozoa are most active and survive for the longest period in vitro at pH of about 7.0 (Mann, 1964). There is a fairly rapid fall of motility and metabolic activity on either side of the optimal pH around 6.5 in ram and bull (Lardy and Phillips, 1939 & 1943; Blackshaw, 1954a & 1960a, and Foote, 1964). It is necessary to have a buffer such as phosphate, citrate or bicarbonate in the media (White, 1969) for pH adjustment. Mann and White (1957) claimed that the production of lactic acid by spermatozoa increased in the presence of phosphate or glycerol.

The presence of sugar (Blackshaw and Emmens, 1951) or egg yolk (Bogart and Mayer, 1950) or/and milk (Foote, 1969) in the diluent reduces the effect of pH or brings it to the neutral point. Leverage et al., (1972) claimed that the characteristics of spermatozoal motility changes when semen is exposed to the buffers as well as after freezing and thawing.

The pH of the normal ram ejaculated semen, is slightly acidic, ranging between 6.6-7 (Hulet and Ercanbrack, 1962; Nalbandov, 1964; Terrill, 1968; White, 1969, and Fraser, 1971a).

Spermatozoa remains motile for the longest period in a media having about the same tonicity as semen. In general they are less readily affected by hypertonic than by hypotonic conditions (Quin et al., 1968b; Quinn and White, 1969, and White, 1969). Ram spermatozoa freeze better in hypertonic than hypotonic diluents (Jones, 1965b; Salamon and Lightfoot, 1969; Ostasko, Bugrov and Zvereva, 1969 and

Salamon, 1970). On the contrary hypertonic diluents were harmful to ram and bull spermatozoa (Blackshaw, 1960a; Saake and Almquist, 1963b; Martin, 1963b & e & 1965a; Nagase and Niwa, 1964; Rao, Sikes and merilan, 1968, and Quinn, White and Voglmayr, 1970) and to fowl semen (Hobbs and Harris, 1963).

Lapwood and Martin (1966) found no harm to ram spermatozoa stored in hypertonic solution of sugars. Martin (1963e) claimed that the combination of fructose and buffer citrate kept bull spermatozoal motility much better than either of them separately. Davis, Bratton and Foote (1963), Benson, Pickett, Lucas and Gebauer (1967) Prasad and Norman (1968) and Yassen and El-Kamash (1972) found that the buffer yolk glycerol extender was superior to the standard citrate yolk glycerol (Stower and Bud-Husaim, 1957; Schindler and Amir, 1961; Emmens and Robinson, 1962, and Marshall, Saake and White, 1968) in storage or freezing of ram and bull semen. Belgium Committee (1954) and Lapwood and Martin (1972) found that citrate buffer depressed ram motility on storage at 37°C or 5°C. Foote and Leonard (1964) found that sodium citrate buffer was superior to phosphate buffer, and the replacement of sodium by glycine prolonged the frozen dog spermatozoal survival. Milovanov and Selivanova (1932), and Ten En Bon (1965) found that the addition of phosphate increased the resistance of ram semen to cold shock. Loginova and Zeltobrjuh (1968b) found no difference between citrate and phosphate containing diluents and sulphate was inferior to both and Kalev, Marinov, Zagorski, Kitchev, Bak'Rdzhiev and Zherkov (1971) found no difference between including or omitting the citrate from the diluent on survival of the frozen ram spermatozoa. Easley et al. (1942) claimed that sugars (glucose), phosphate and calcium containing diluents were markedly harmful to bull spermatozoa during storage.

Sugars

The presence of certain non-electrolytes in the form of sugars, in the diluents improve the revival rate of mammalian spermatozoa during storage and after freezing. The preferable sugar in the diluent was 11% lactose (Fraser, 1962; Aamdal and Andersen, 1968b; Sainsbury, 1968; Loganova and Zeltobrjuh, 1968a; Salamon, 1968; Salamon and Lightfoot, 1969; Anderson and Aamdal, 1972, and Polge, 1974), or fructose (Martin and Emmens, 1961; Curtis, Forteath and Polge, 1961; Choong and Wales, 1963; Martin, 1965b, and Jones, 1965b) or arabinose (Emmens and Blackshaw, 1950 & 1955; Blackshaw and Emmens, 1953; White et al., 1954; Blackshaw, 1955a; First, Henneman and Williams, 1957; Emmens and Martin, 1961, and Martin and Watson, 1973), or glucose (Jones and Martin, 1965; Martin, 1966 & 1968, and Salamon and Visser, 1972).

Salamon (1968 & 1970), and Salamon and Lightfoot (1969) found that revival of ram spermatozoa after freezing in egg diluents containing sugar was best with lactose followed by raffinose, glucose, and fructose, but when tris base was included in the diluent the reverse order of the sugars' importance was the result (Salamon and Visser, 1972). In addition Visser and Salamon (1974) found that in freezing boar semen, survival of spermatozoa declined as the tris concentration increased in a diluent containing sugar, particularly lactose.

Sugar containing diluents were superior to those containing citrate in surviving spermatozoa after freezing (Emmens and Blackshaw, 1950; Blackshaw, 1955a; First et al., 1957; Nagase and Niwa, 1964; Salamon and Lightfoot, 1969, and White, 1969).

Nagase, Graham and Niwa (1964) and Nagase and Graham (1964) found

no significant differences between extenders containing glucose, raffinose, lactose or their combinations.

Antibiotic

Dauzier (1956), Melrose, Stewart and Bruce (1958) and Salamon and Robinson (1962) claimed that the inclusion of antibiotic in the milk diluents increases the fertilizing capacity of the bull and ram spermatozoa when stored in unfrozen state. On the contrary, Almquist, Thorp and Knodt (1946) and Emmens and Blackshaw (1956) found that treatment of semen with 1000 I.U./ml of antibiotic (penicillin or streptomycin) was harmful on storage in vitro.

Park, Melrose, Stewart and O'Hagan (1964) found that 500 µg/ml of streptomycin was inferior to tetracycline (25-50 g/ml) in controlling the bacterial growth in bull and boar semen stored at 5°C. Almquist, and Zaugg (1974) found no significant difference among antibiotic combinations on the fertility of frozen bull semen, and Hurst (1953) found no difference between their inclusion or their absence.

Egg yolk

Egg yolk has been recommended as a common ingredient of semen diluent since Lardy and Phillips (1939). Egg yolk or its derivatives such as lipoprotein or lecithin have been found to be a good protective agent to the spermatozoa against cold shock during cooling and preservation at low temperature as well as freezing (Easley et al., 1942; Lasley and Mayer, 1944; Mayer and Lasley, 1945; Bogart and Mayer, 1950; Kampschmidt, Mayer and Herman, 1953; Blackshaw, 1954a; Miller and Vandemark, 1954; White et al., 1954; Blackshaw and Salisbury, 1957; Sherman, 1962; Quinn and White, 1966b; Entwistle and Martin, 1972; Masuda and Nishikawa, 1972; Jones and Martin, 1973; Watson and Martin,

1973a & 1974; Visser and Salamon, 1974; Pace and Graham, 1974, and Watson, 1975a). Egg yolk in proportion of 6.5-13% (Entwistle and Martin 1972) 15% (Salamon and Lightfoot, 1969), 16-24% (White et al., 1954) 24% (First, Henneman, Magee and Williams, 1961) or 25% (Miller, and Vandemark (1953) was sufficient to protect ram and bull spermatozoa during freezing. Blackshaw and Emmens (1953) and Emmens and Blackshaw (1955) claimed that 50% egg yolk improved the revival rate of frozen ram spermatozoa. On the contrary Foote and Leonard (1964) found that 20% of egg yolk was superior to 50% in storage of dog semen, and First et al., (1957) found no difference between 30, 37.5 and 50% egg yolk in freezing ram semen. The inclusion of egg yolk in the diluents increased the proportion of the spermatozoa that retained motility after cooling to 5°C and achieved reasonable post thawing survival in ram (Sainsbury, 1968, and Salamon and Lightfoot, 1969), bull (Lanz, Pickett and Komarek, 1965), human (Norman, Goldberg, Porterfield and Johnson, 1960) and boar (King, 1971). Polge and Butler (1973) and Butler and Roberts (1975) found that the addition of certain phospholipids protect boar or ram spermatozoa against cold shock.

Egg yolk citrate diluents were superior to those based on milk for dilution of ram semen (Salamon and Robinson, 1962) bull semen (Underbjerg, Davis and Spangler, 1942) and both (Blackshaw, 1955b).

First et al., (1961a) noted a tendency toward decreased survival of ram spermatozoa frozen in diluent based on milk as the level of egg yolk was increased (from 2-25%). Bogart and Mayer (1950) got the better results in storage and Nagase, Niwa, Yamashita and Irif (1964b & c) in freezing bull semen in pellet, with yolk sugar diluents than yolk citrate. Nagase, Yamashita and Sirie (1968) found that egg yolk and lactose or

raffinose protected bull spermatozoa during freezing without glycerol.

Milk

Satisfactory results were obtained after storage and freezing ram and bull semen in diluents based on various proportion of milk, i.e. boiled, pasteurized, homogenized skimmilk or reconstituted milk (First, et al., 1961a; Choong and Wales, 1962; Jones, 1967, 1968 & 1969a, and Jones and Foote, 1972).

Diluents based on milk and sugars were superior to egg yolk citrate diluents for storage as well as freezing ram semen (Dauzier, 1956; Emmens and Robinson, 1962; Morozov, 1964; Jones and Martin, 1965; Branny, Pilch and Wierzbowski, 1966; Colas, Courot and Ortavant, 1972, and Barlow, Pryce-Jones and Reed, 1974), and bull (Melrose et al., 1958, and Curtis et al., 1961), but Foote and Leonard (1964) claimed that milk extenders were less satisfactory for freezing dog semen. Martin (1972) found the inclusion of egg yolk (3%) in the milk sugar diluent was necessary for high dilution (10-30 folds) of the ram semen. Thacker and Almquist (1953), Blackshaw and Salisbury (1957), Dauzier, Mesnil and Buisson (1958), Blackshaw (1960a & b), Salamon and Robinson (1962), Lunca (1969) and Patt and Nath (1969) found that there was no significant differences between egg yolk diluents and skim milk, but fresh milk was inferior to both on freezing ram semen. Blackshaw and Emmens (1953), Emmens and Blackshaw (1955), Szumowski, Markovic and Cano (1956), Melrose et al., (1958), Hill et al., (1959), Curtis et al., (1961), Melrose (1961), Stower and Cembrawicz (1961), First et al., (1961a), Jones and Martin (1965), Jones (1965b), Entwistle and Martin (1972) and Vinha and Coubrough (1972a & b) found that synthetic diluents containing sugar and electrolytes, together with small proportions of preparations from milk

or egg yolks or both protected bull and ram spermatozoa during freezing and thawing, and were comparable or superior to either milk or egg yolk separately.

Storage of bull spermatozoa at 4°C in diluents containing egg yolk citrate and milk led to the ageing of the spermatozoa with resultant decline in fertility, accompanied by prenatal losses (Salisbury and Flerchinger, 1967a) or irregular estrous cycle (long) after unsuccessful insemination (Salisbury and Flerchinger, 1967b), and the ageing was worse in frozen semen, with resultant early embryonic death at the stage of the zygote (Salisbury, 1967).

Foote and Dunn (1962) found that the addition of catalase to the diluents did not improve the spermatozoal activity in bulls.

Cryoprotective

It is generally agreed that glycerol, dimethylsulphoxide (DMSO) or other cryoprotective is essential to protect the spermatozoa against the freezing injuries since the findings of Polge et al., (1949) and Smith and Polge (1950a & b).

Glycerol is toxic to the spermatozoa especially at high concentration (White et al., 1954; Miller and Vandemark, 1954; O'Connor and Smith, 1959; Melrose, 1962; Sherman, 1963; Drevius, 1963; King and Macpherson, 1966; Fraser, 1968; Berndtson and Foote, 1969; Sanford, King and Macpherson, 1972, and Visser and Salamon, 1974) by decreasing the oxygen uptake with their immobilization pre and post freezing. Dimethyl sulphoxide has an equal protective action to glycerol but it is more toxic to the spermatozoa than glycerol (Sherman, 1964; Jones, 1965a & b; Richardson and Sadleir, 1967, and Hood, Foley and Martin, 1970), and to bone marrow tissue (Porterfield and Ashwood-Smith, 1962).

The presence of glycerol in the diluent depressed the spermatozoal fertility of the frozen semen either when the glycerol percentage was high or was not removed from the thawed semen of the bull (Swanson, Thigpen and Stanfill, 1962), fowl (Polge, 1951, and Shaffner, 1964) and depressed the spermatozoal motility in dogs (Martin, 1963c) and boar (Neville, Macpherson and King, 1970, and Sanford et al., 1972). On the contrary, Polge and Rowson (1952a & b), Holt (1953b & c), Hafs and Elliott (1954) and Almquist (1959) found that glycerol improved the spermatozoal fertility of bulls and Brown and Harris (1963) of fowl.

It has been found that low percentage of glycerol (2%) increased the respiration rate in ram (Mann and White, 1956, and O'Shea and Wales, 1966), and human spermatozoa (Ackerman, 1970), while such quantities of glycerol do not affect bull spermatozoa (O'Shea and Wales, 1966).

It has been found that DMSO (Lovelock and Bishop, 1959, and Dougherty, 1962) as well as pyridine N-Oxide (PNO) (Nash, 1961 & 1962) produced less damage to the tissue culture, human and bovine red blood cells during freezing than glycerol, due to their higher permeability through the cell membranes (Ashwood-Smith, 1961). On the other hand the protective efficiency of the glycerol (Lovelock and Bishop, 1959, and Richardson and Sadleir, 1967) or of the ethylene glycerol (Salamon, 1968) to bull, man and ram spermatozoa were superior to DMSO.

The efficiency of DMSO in preserving ram spermatozoa during incubation or storage at 5°C was equal to glycerol (Jones, 1965a) but inferior to it during freezing (Jones, 1965b). The addition of low level of DMSO (1.5%) along with glycerol (7%) was superior to either 3-9% DMSO or 7% glycerol in freezing ram semen (Jones, 1965b). Snedeker and Gaunya (1969) found that 2-2.5% DMSO with no more than 5% glycerol

gave the highest post freezing motility of bull semen extended in milk diluent. Similar success in freezing bone marrow has been achieved by Porterfield and Ashwood-Smith (1962) following the combination of DMSO and glycerol of 2.5% each or 10% of each separately. On the other hand Visser and Salamon (1974) did not succeed in freezing boar semen following the combination of glycerol and erythritol at various levels.

Polge et al. (1949) found 5% glycerol to be highly toxic to the rabbit spermatozoa, while fowl spermatozoa tolerated up to 20%, but were infertile unless the glycerol was reduced to 2%.

The proper glycerol level to provide reasonable protection to the spermatozoa during deep freezing of ram semen was 3.5% (Platov, 1965 & 1966), 2.4 or 4% (Lightfoot and Salamon, 1969a), 7% (Boureanu and Negoita, 1971), 7.5% (Jones, 1965b), or not less than 6% and not more than 10% (6-8%) of glycerol (First et al., 1961a) while Colas (1975) found that 4% was superior to 2%; for bull semen 5% (Almquist and Wickersham, 1962), 7% (Saroff and Mixner, 1955), 10% (Rowson and Polge, 1953; Bruce, 1953, and Holt, 1953a) or 10.7% (Wiggin and Almquist, 1975a), and for boar semen 1-2% (Polge and Butler, 1973) or 4.5-7.5% (Salamon, Wilmut and Polge, 1973). In addition Wilmut et al. (1973) found that glycerol requirement for freezing boar semen was associated with cooling time of the diluted semen to 4°C (for 1 step) and the equilibration time (for 2 steps) the shorter equilibration, the higher glycerol level requirement and vice versa, e.g. 4, 6 and 8% glycerol with 5-10 minutes, 30 seconds, and 5-3 seconds respectively preceded by 1-2 hours cooling at 5°C.

Martin (1963c) found no significant difference between 8% and 4% glycerol, but 8% was slightly better, in freezing dog semen.

Curtis et al. (1961) also found no significant differences between 4 and 8% glycerol, but 4% was marginally superior in freezing bull semen.

Choong and Wales (1964) and Vinha and Coubrough (1972a) found that 6% glycerol was optimal in freezing bull semen, but levels as low as 2% (Salamon, 1970) or 1% (Nagase et al., 1964a, and Salamon, 1968) in the presence of lactose were still giving a reasonable result in freezing bull semen. Fraser (1968) and Colas (1975) in freezing ram semen found that, above 4%, glycerol had a toxic effect on spermatozoa and altered their morphological characters (bend tails).

Almquist and Wickersham (1962) found that in freezing bull semen the survival of spermatozoa was increased with each decrease in glycerol level from 20 to 5% in skim milk diluent. Nagase et al. (1964b) and Fraser (1968) found that 3.5% glycerol in egg yolk lactose gave a good result in freezing bull and ram semen respectively.

Saroff and Mixner (1955) and First, Henneman and Magee (1959a) found that as the egg yolk increased the glycerol percentage has to be increased in order to get the best survival of bull and ram spermatozoa, after freezing. They suggest that the egg yolk in some manner tied up part of the glycerol, making the requirement for glycerol higher as the egg yolk contents increased. Lightfoot and Salamon (1969a) found that the effect of glycerol concentration on the diluted semen on the post thawing motility of the ram spermatozoa, varied with different diluents (lactose or raffinose, with or without sodium citrate), methods of dilution (one dilution at 30°C, two additions at 30°C or one addition at 30°C and second addition at 5°C), and time of equilibration at 5°C (1, 4 or 7 hours). Best results were obtained with 2.4 or 4% glycerol and sugar and short equilibration time, but in the presence of sodium citrate or dilution by a single addition at 30°C or long equilibration, the glycerol percentage has to be higher (5.6%). In addition, Nagase

and Niwa (1964), Nagase et al. (1964a & b), Salamon (1968), and Salamon and Lightfoot (1969), found that in freezing ram or bull semen optimum glycerol percentage varied with different sugars, i.e. 3% with lactose, 5% with raffinose or glucose and 7% with fructose. Nagase et al. (1964b), Nagase (1968) and Salamon et al. (1973) found that the high molecular weight of the sugar component of the diluent led to reduced requirement for glycerol or any other cryoprotective in freezing the semen of the domestic animals.

Salamon (1968) claimed that lactose can give reasonable survival with only 1% or even without glycerol. Emmens and Blackshaw (1950) claimed that freezing of ram semen in 7-10% glycerol containing diluent was not satisfactory unless 1.25% arabinose, rhaminose or xylose had been added.

Bostrom and Rubin (1973) found that there was no difference in the abnormal morphology between human spermatozoal samples frozen with or without glycerol.

In addition glycerol percentage requirement in freezing the ram semen (Hill et al., 1959) as well as bull semen (White et al., 1954) differed in different individuals.

Nagase et al. (1964a) found no significant differences between 1-7% glycerol in freezing bull spermatozoa, but there was a decrease in motility after thawing as glycerol concentration decreased below 1.95%. There was no difference in the post thawing survival of ram spermatozoa (Nagase and Niwa, 1964) after freezing in 3.5, 7, or 7.5% glycerol and of bull spermatozoa (Hill et al., 1959) after freezing in 3.5, 5.75 or 7% glycerol.

The post thawing activity of the spermatozoa increased as glycerol level increased to 6-8% in freezing ram semen (First et al., 1957 & 1961a),

as well as bull semen (Miller and VanDemark, 1953 & 1954).

Method of dilution

The suitable temperature at which the glycerol has to be added varies with species and the diluent components.

Glycerolization at 4-5°C was preferable with milk diluted ram spermatozoa (Blackshaw and Emmens, 1953; Blackshaw, 1960b, and Colas, 1975), as well as bull spermatozoa (Almquist, 1959) and in both species (Blackshaw, 1955b).

Bull semen diluted with egg yolk citrate survived better when glycerolized at 29°C (McFee, 1959, and Melrose, 1961) than at 5°C. However, bull spermatozoa tolerated both temperatures, but 5°C was preferable (Polge, 1953; Miller and VanDemark, 1954; Blackshaw, 1960a& b; Almquist and Wickersham, 1962; Choong and Wales, 1963, and Martin, 1965b). A similar result was obtained in the dog (Foote and Leonard, 1964). Branny et al., 1966, also found that ram semen tolerated the addition of glycerol at either 32°C or 3°C.

The addition of the glycerol by one step at 5°C (Entwistle and Martin, 1972) or at 30°C (Lightfoot and Salamon, 1969a) is preferable in freezing ram semen. Sherman (1962b) found that bull spermatozoal survival decreased as the temperature of glycerolization increased from 5 to 21 to 37°C but in contrast human spermatozoa were not affected. Holt (1953b) found that the optimum glycerol addition time was different in different bulls. Bruce (1953) and Entwistle and Martin (1972) found no significant difference between immediate or slow addition of glycerol at 5°C in ram and bull spermatozoa. On the contrary Miller and VanDemark (1954), Stower and Cembrowicz (1961), Feredean and Bragaru (1963 & 1964) and Choong and Wales (1964) recommended the slow way of

glycerolization at 5°C. Emmens and Martin (1961) and Lightfoot and Salamon (1969a) claimed that the type of sugar in the diluent appears to determine whether the glycerol should be added at 30°C with one step or after cooling to 5°C with two steps. In addition Almquist and Wickersham (1962) prefered three steps in glycerolization of bull semen. Wilmut, Salamon and Polge (1973) in freezing boar semen found that the addition of glycerol to the boar semen at 5°C depended on glycerol concentration, the time of cooling to 5°C and the equilibration time. Page, Gebauer, Snedeker and Gaunya (1968) found that the addition of the DMSO in four equal parts at 15 minutes interval was superior to one part in freezing the bovine spermatozoa.

Dilution rate

The rate of semen dilution, actually depends on the spermatozoal density (Holt, 1953a), spermatozoal activity at the time of collection as well as the diluent composition and the storage time (Salamon, 1968).

Excessive dilution (over 10 fold) of ram semen is harmful to the spermatozoa by reducing their motility and fertility (Blackshaw, 1953, and Martin, 1968), and causes a marked decrease in their fructolysis and respiratory activities (Mann and Lutwak Mann, 1948).

Excessive dilution of the ram semen lowersthe fertilizing ability of the spermatozoa after storage at 4°C (Schindler and Amir, 1961; Martin, 1968; Sahni and Roy, 1968; Antonjan and Kamaljan, 1970, and Martin and Watson, 1973), as well as after freezing (Lightfoot and Salamon, 1969a & b, and Salamon and Lightfoot, 1970). Blackshaw (1953) found that bull and ram spermatozoa lose their motility at relatively high dilution with diluent free of egg albumin or seminal plasma.

The dilution rates of 1:4-8 seemed to be the most suitable rate for freezing ram semen (Platov, 1965; Aamdal and Andersen, 1968b; Fraser, 1968; Lightfoot and Salamon, 1969a; Jones 1969b, and Andersen and Aamdal, 1972).

B) Equilibration time

This is the storage time of the diluted semen, through which there will be an equilibration in the osmotic pressure of the diluent and of the spermatozoa.

The optimum equilibration period differs in different countries and among centres in the same country. At the same centre, it is also different from sire to sire (Nishikawa, 1964) and between ejaculates of the same individual as found by Choong and Wales (1963), and Brown and Harris (1963) in the fowl as judged by the fast thawing motility and fertility of the frozen semen samples.

Usually equilibration times vary according to the storage temperature, cooling rate to the storage temperature, dilution and the species.

Slow cooling of the diluted ram semen to 0-2^{o}C was preferable (Kuprijanova 1962, and Ten En Bon, 1966). Cooling for 1.5 hours (Stewart, 1961), 1-2 hours (Wilmut et al., 1973), 2.5 hours (Patt and Nath, 1969), 1-3 hours (Jones, 1969b), 2-3 hours (Blackshaw, 1955a & 1958a), 2-4 hours (Feredean and Bragaru, 1963), 4 hours (Hill et al., 1959, and Benson et al., 1967), 4.5-5 hours (Jones, 1965b) or 6 hours Martin, 1965b) has to be followed by short equilibration in freezing ram, bull or boar semen. In addition, Colas (1975) in freezing ram semen and Wilmut et al. (1973) in freezing boar semen found that

equilibration time depended on the glycerol percentage, the shorter equilibration the higher glycerol percentage requirement and vice versa, e.g. 5-30 seconds, 30 seconds, 5-10 minutes, equilibration, and 8, 6, 4% glycerol in boar, or 20 minutes, 150 minutes equilibration and 4, 2% glycerol in ram respectively.

Polge (1974) found that storage of the diluted spermatozoa in vitro required long time to get acclimatization to the new environment, so he advised to prolong the equilibration time in freezing bull semen (overnight). Saroff and Mixner (1955) found that the longer equilibration (up to 18 hours) the better the survival rate for bull semen. Semen diluted with egg yolk and glycerol needs a long equilibration time (overnight) as found by Polge and Rowson (1952a & b), Cragle et al., (1955), Blackshaw (1955b) and Nagase and Niwa (1964). On the contrary Martin (1966) and Jones and Martin (1973) in freezing ram semen and Wiggin and Almquist (1975a) in freezing bull semen found that shorter equilibration is necessary.

Martig and Almquist (1966) found that 0.5 and 4 hours equilibration were superior to 2 and 18 hours, while Wiggin and Almquist (1975a) claimed that 2 hours was superior to 1, 4, 8 or 16 hours in freezing bull semen. Blackshaw and Emmens (1953), Blackshaw (1955b) and Emmens and Martin (1957) found that when arabinose or fructose (Martin 1963b) was used for freezing bull semen, glycerol equilibration was not necessary or at the most for 0.5 hour, but the fertility was better in those equilibrated for 18 hours. On the contrary, the inclusion of sugars in the diluents required long equilibration time (up to 18 hours) in order to obtain better results according to Martin and Emmens (1961) and Martig and Almquist (1966).

Leverage et al. (1972) found that primate including human spermatozoa froze well without equilibration or just 0.5 hours at 37°C while bovine spermatozoa did not freeze well unless equilibrated for 18 hours at 5°C. Jones (1969b) found that ram spermatozoa froze best, as judged by their motility, after incubation at 30°C for 2 hours followed by equilibration at 5°C for 20 minutes.

Berndtson and Foote (1972) found that bull spermatozoa showed an improvement in their motility following freezing and thawing after 10 seconds equilibration in comparison with 2, 6, 30 and 360 minutes. They claimed that part of this difference can be attributed to the harmful effect of glycerol on spermatozoa during the longer exposure period at 5°C before freezing and part to avoidance of intracellular ice formation. There was no difference with equilibration time of bull spermatozoa for 18 and 1 hour (First et al., 1959b) or 18 hours and 5 minutes (Sherman, 1957), but Emmens and Martin (1961) found 18 hours was superior to 1 hour. Martin (1963b) found no difference in the post thawing motility after 4 or 8 hours equilibration of bull semen, but, following subsequent incubation, motility remained highest in the 4 hour samples.

Polge and Jakobsen (1959) found that bull spermatozoal survival was always lowest in samples which were equilibrated for 3 hours following the initial dilution but there was a gradual increase in the survival rate as the equilibration extended up to 7-8 hours, especially after rapid freezing and the survival rate declined in those samples frozen after 24 hours.

Polge (1953) found that motility score was higher after 0.5 than 18 hours but with low fertilizing capacity in bulls.

Roussel, et al. (1974) observed that the best equilibration time varied with height of the straws above the surface of the liquid nitrogen, i.e. the higher the longer.

In general equilibration of the mammalian spermatozoa with diluents containing glycerol for 18 to 24 hours (overnight) prior to freezing is no more beneficial for the spermatozoa than 0-5 hours equilibration and in some cases it might be detrimental (White et al., 1954; Blackshaw, 1955a & 1958a; Emmens and Martin, 1957; Blackshaw, Emmens, Martin and Heyting, 1957; Hill et al., 1959; Fraser, 1973, and Bielanski, 1973).

C) Freezing

It is believed that the formation of large intracellular ice crystals after slow freezing and thawing, disrupt the vital cellular components and cause the death of the cell (cited by Salisbury and Van Demark, 1961b).

On the other hand small ice crystals might be the result after fast freezing and thawing, but too rapid freezing may cause salt concentration to be increased as the water freezes. This increases the osmotic pressure which may be damaging to the proteins and lipoproteins of the spermatozoal cells (cited by Foote and Trimberger, 1969).

Rapid cooling or warming of the ram semen between freezing point and body temperature (0-39°C) was recommended by Chang and Walton (1940) and Walton (1957), to avoid the spermatozoal cold shock.

Similarly, rapid freezing was recommended in the form of pellet by Nagase and Niwa (1963a & b) or in the form of straws by Cassou (1964) and Jondet (1964).

Direct dropping of the diluted semen into liquid nitrogen was deleterious and gave unsatisfactory results in ram (Salamon, 1967, 1968 & 1970) and bull (Nagase et al., 1964b, and Nagase and Tomizuka, 1968).

Salamon (1967) observed that ram spermatozoa unlike those of the bull did not maintain their fertilizing capacity after semi rapid and rapid freezing in synthetic straws and pellet form. Semi rapid freezing gave similar results to the rapid freezing method in freezing bull semen (Nagase and Graham, 1964) and in ram semen (Salamon, 1970). Hill et al. (1959), Sherman (1962a), Bruemmer, Eddy and Duryea (1963) and Nagase and Niwa (1964) found no significant differences between slow and rapid freezing of ram, bull or human, but the mean percentage recovery for the slow rate of freezing was higher than that for the more rapid rate of freezing.

Rowson (1957) claimed that slow cooling of the semen to $-10^{o}C$ followed by rapid freezing was necessary to avoid the dangerous point (-10 to $-35^{o}C$) and to reduce the opportunity of cold shock to the spermatozoa.

On the other hand, slow freezing was preferable and the spermatozoal survival was better in ram (Emmens and Blackshaw, 1950; Kuznecov and Kuprijanova, 1959; Kuprijanov, 1962; Feredean and Bragaru, 1963 & 1964; Jones, 1969b, and Entwistle and Martin, 1972), bull (Smith and Polge, 1950a; Polge, 1953; Stower, 1953, and Martin, 1965b), and dog spermatozoa (McFee, 1959). Cooling rate of $1^{o}C$/minute to $-79^{o}C$ (Smith and Polge, 1950a) or $1-4^{o}C$/minute, between $5^{o}C$ and $-20^{o}C$ (Miller and VanDemark, 1954) and then followed by freezing within 40 minutes (Fraser, 1962) were superior to rapid cooling.

Birillo and Puhaljskii (1936), Gladcinova (1937), Easley et al. (1942), Smith and Polge (1950a), Blackshaw (1958b), McFee (1959), Wales and White (1959), and Martin (1965a) found that most of the domestic animals spermatozoa except man (Sherman, 1962b) were relatively insensitive to rapid temperature changes between 38°C to 5°C, especially from 15°C to 5°C, but ram spermatozoa were more susceptible to cold shock during cooling than that of the bull (Choong and Wales, 1962; Misra and Sengupta, 1965, and O'Shea and Wales, 1967).

The problem of thermal shock may be largely overcome by freezing the semen in small sphere or pellet or mini-straws, i.e. less than 1ml (Nagase and Graham, 1964; Sainsbury, 1968; Salamon, 1973; Polge, 1974 and Pickett and Berndtson, 1974).

Anderson, Byers and Pickett (1969) found that the survival rate of stallion semen after freezing in 1ml. ampoules was superior to 10ml tubes. On the contrary human semen survival was better after freezing in bulk than small quantities (Parkes, 1945).

Nagase and Niwa (1964) found that there was no difference in survival rate due to pellet size when yolk glucose diluent was used, but the smaller size was better with yolk citrate diluent.

There was a great disruption of spermatozoal motility and metabolism after freezing as well as storage at low temperature (-79 to -196°C) of some mammalian spermatozoa (ram, bull, boar, rabbit) as found by White et al. (1954), Quinn et al. (1969), O'Shea (1969b), Darin et al. (1973), Darin-Bennett et al. (1973), and Murdoch and O'Shea (1973). Graves (1968) and Ackerman (1968 & 1970) claimed that some matabolic activity takes place in ram and human spermatozoa during their storage at -196°C.

D) Storage

Storage of ram, bull or human semen at -196°C has been found preferable to -79°C (Larson and Graham, 1958; Kelly and Hurst, 1964; Clegg and Pickett, 1966; Freund and Wiederman, 1966, and Entwistle and Martin, 1972), while Rowson and Polge (1953) found no difference between the two temperatures on freezing bull semen. Morozov (1964) found that freezing and storage of the ram semen at -79° or -196°C was superior to -20°C.

Storage of semen at 5°C or higher caused a kind of ageing which led to decline in spermatozoal motility (Singh and Sadhu, 1966) and loss of the seminal proteins (Leslie and Quinlivan, 1968) and the loss of DNA content of the spermatozoa (Salisbury, Birge, De la Torre and Lodge, 1961, and Blackshaw and Salisbury, 1972), followed by decline in their fertility (Aamdal, 1952; Campbell, 1953; Hewetson, 1955; Dauzier et al., 1958; Dott, 1964; Ten En Bon, 1966; Hart and Salisbury, 1967, and Welch and Shannon, 1970).

Frozen ram semen sometimes presented a higher motility percentage after 2 week's storage than after shorter storage time, but there was no more change in motility on further storage (Hill, et al. 1959, and Sainsbury, 1968). Freezing as well as storage of ram spermatozoa decreases the fertilizing ability of the ram spermatozoa in spite of their satisfactory post thawing motility as found by Emmens and Blackshaw (1955), First et al. (1959b & 1961a & b), Kuprijanova (1962), Morozov (1964), Salamon (1967), Martin (1968) and Loginova and Zeltobrjuh (1968a). The same findings have been observed with stallion semen (Barker, 1962), boar semen (Pursel, Johnson and Gerrits, 1969) and bull semen (Martin and Emmens, 1958).

E) Thawing

i) Thawing temperature

Thawed frozen semen, neither survives for as long as unfrozen semen, nor refreezes well, so the basic policy is to use thawed semen as soon as possible (Foote and Trimberger, 1969).

Robbins et al. (1972) claimed that the critical test for evaluating the efficiency of the thawing temperature is the incubation of the spermatozoa at 37°C. Robbins et al. (1972) and Colas (1975) found that the spermatozoal motility decreased as the incubation time at 37°C increased.

Chang and Walton (1940) found that there was no temperature shock when ram spermatozoa were raised from lower to higher temperature, and the faster the better.

Generally, rapid thawing as well as freezing of the spermatozoa is desirable to pass the dangerous or the critical point (-12 to -25°C) through which the ice crystalization is associated with intracellular damage (Luyet and Hodapp, 1938; Rowson, 1953; Lovelock and Polge, 1954; Sherman, 1955; Polge, 1957; Polge and Jakobsen, 1959; Platov, 1966; Sainsbury, 1968, and Ostasko et al., 1969).

There is real need to establish the best thawing temperature or/and medium. Most investigators findings differ according to the different species, and individuals (Dunn, Hafs and Young, 1953). Some prefer slow thawing rate at low temperature (0-5°C) or medium (10-30°C) and others fast thawing rate at body temperature or higher up to the boiling point of water.

Fast thawing (35-75°C) was superior to slow (4°C or 20°C) and

led to an increase in the percentage motility as well as the unstained spermatozoa of ram semen (Aamdal and Andersen, 1968b and Andersen and Aamdal, 1972), bull (Aamdal and Andersen, 1968a; Robbins et al., 1972; Almquist and Wiggin, 1973a, and Wiggins and Almquist, 1975a) and in both species (Blackshaw, 1955b). In addition Wiggin and Almquist (1975a) found that the acrosomal damage decreased as the thawing temperature increased from 35 to 95°C.

Aamdal and Andersen (1968a & b) and Almquist and Wiggin (1973a) found that 75°C/10 seconds or 76°C (Wiggin and Almquist, 1975b) to be the optimu, thawing temperature but boiling water (95-100°C) was superior to 35°C and both were superior to 5°C. Robbins et al. (1972) found an increase in the acrosomal damage of bull spermatozoa after thawing at 5°C, followed by incubation at 37°C. Rowson (1953) found no significant differences between 100, 40 and 5°C thawing temperature on the percentage of live spermatozoa in bull semen. Platov (1965, 1966 & 1968) and Salamon (1968) found that 45, 40 or 30°C in thawing frozen ram semen, Salamon et al. (1973) found 37, 50 or 60°C (the higher the better) in thawing the frozen boar semen and Roussel et al. (1974) found 43.5°C in thawing frozen bull semen were superior to 20 or 5°C. Anderson et al. (1969) found 38°C was superior to 26, 15, and zero in thawing stallion semen. On the other hand Miller and Van Demark (1953 & 1954) claimed that 5°C thawing temperature for frozen bull semen was superior to 38°C. Watanabe (1968) found that thawing and incubation of the frozen fowl semen at 20°C gave a significantly higher motility than at 30°C or 37°C. Zakrzewska (1962) examined the process of thawing bull frozen semen at room temperature (15-18°C) and at 40°C. The motility of the spermatozoa after thawing was about the same at the end of both processes, but after

0.5 hours was better in those samples which were thawed at room temperature (Belgium Committee, 1954). Hafs and Elliott (1954), and Pickett, Hall, Lucas and Gibson (1965) claimed that there were no significant differences between 1-5°C and 40°C thawing temperature and both were superior to 15-20°C in thawing bull frozen semen. Boyd and Hafs (1968) claimed that 0°C is preferable because it is more easily maintained constant than any other temperature.

ii) Thawing medium

The redilution of the thawed ram semen (Bravo and Cerezo, 1964) in saline solutions (First et al. 1961a) or sugars (Jones and Martin, 1965; Lightfoot and Salamon, 1969a & b, and Salamon and Brandon, 1971) and the addition of yolk citrate (Visser and Salamon, 1974) or yolk glucose (Salamon, 1973) to the thawed boar semen improved the post thawing motility of their spermatozoa. Thawed ram semen survived in Krebs-Henseleit ringer solutions better than in milk (Jones, 1969b) and both diluents were superior to egg yolk citrate (Jones and Martin, 1965). O'Shea (1969a) found that thawed ram and bull spermatozoa survived well at 37°C incubation after the addition of 5-15% potassium chloride. Nagase et al. (1964a), Page et al. (1968a), Salamon (1968 & 1970) and Butler (1974) found no significant difference between various thawing media (sugar, egg yolk, milk, citrates or chloride) on the post thawing survival in bull and ram semen. Salamon et al. (1971 & 1973) and Salamon (1973) found that post thawing motility was poorer after dilution than without (control) in the case of the boar.

Adler, Jespersen, Meding and Rasbech (1968) found that the addition of 1ml homogenized sterile milk to the insemination dose of bull semen was beneficial.

Desjardins and Hafs (1962) suggest that it is advisable to re-extend thawed bull semen in a medium different from that used for freezing of the same sample. They found that egg yolk thawing solution was inferior to the C.U.E. (Cornell University Extender). Pursel and Johnson (1975) claim that thawing of the frozen boar semen by adding the thawing solution prewarmed up to 50°C to the 10ml frozen pellet is the best way.

iii) Dilution rate of the thawed semen

Lightfoot and Salamon (1969a & b) and Salamon and Brandon (1971) claimed that the higher dilution prior freezing of ram spermatozoa required the lower post thawing and vice versa. The preferable dilution rate during thawing ram semen was 1:3 (Salamon, 1968 & 1971, and Salamon and Visser, 1972) or 1:4 (Salamon, 1967).

Lightfoot and Salamon (1969b) found that the volume of the pellet can be increased from 0.03 to 0.86ml. without reducing the post thawing survival rate of the ram spermatozoa. Also there was no evidence of effects due to interaction between pellet volume and either diluent composition or glycerol concentration on post thawing spermatozoal survival.

VI MATERIALS AND METHODS

1. SEMEN COLLECTION

a) Source

Semen samples were obtained from Cheviot, Suffolk and Border Leicester rams aged between one and $2\frac{1}{2}$ years, used for breeding on the farm of the Royal (Dick) School of Veterinary Studies, Field Station, Easter Bush, Roslin, Midlothian. A few samples were also obtained from rams referred to the Field Station clinic for fertility examinations. A general examination and a careful clinical examination of the genitalia of each ram was carried out, (figure 1 and 2) and where any abnormality was found, the ram was rejected for semen collection.

b) Frequency of Collection

Usually semen was collected once weekly or every second week, according to the response of the available rams. On three occasions twice weekly collections were taken from some rams.

c) Procedure:-

All collections were made employing the Plectron electro-ejaculator, which comprises a transformer to convert the standard 230V AC, 50 cycle electricity supply to an output of variable voltage(0-15 V.AC.) and frequency(20-40 cycles), and a bipolar electrode in the form of a rectal probe, 17x3.5cm.

During semen collection the ram was restrained on its side on the ground (figure 3). The rectal probe was lubricated sparingly with water

soluble K-Y jelly and inserted to its full length into the rectum and clear of the anal sphincter. The preputial orifice was cleaned with surgical gauze and a smooth lipped 10ml. graduated tube inserted and held in the prepuce during collection (figure 4).

During semen collection the standard procedure was to commence stimulation at 1-3 volts for few seconds followed by a short interval of rest. Stimulation was repeated until erection of the penis and ejaculation occurred. This usually required 5-10 stimulations. Where there was no ejaculatory response the voltage was gradually increased, the stimulation time, and the rest interval shortened, until a satisfactory collection was obtained.

After semen collection was completed, the semen samples were identified by writing the breed and the number of the individual ram on the tube, then placed in a vacuum flask at 37°C, or cork container to protect the spermatozoa from the effect of light and sudden temperature changes during their transportation from the farm to the laboratory, which took about 15-30 minutes, according to the number of collections as well as the ram reaction. In the laboratory the tubes containing the semen were held in a water bath at 37-39°C, during evaluation and processing.

2. SEMEN EVALUATION

a) Macroscopical examination

The volume of the semen was immediately recorded from the graduation on the collecting tube, and the gross appearance noted.

b) Microscopical examination

i) Spermatozoal motility

a) Mass activity.

The assessment of spermatozoal motility was carried out by placing a small drop of semen on a glass slide on a warm stage at 37-38°C, (Bane, 1952) and examining microscopically at magnification x150. The spermatozoal wave activity was scored from zero (no motility) to four (very strong waves) after the methods of Blom (1946); Emmens (1947); Cragle et al. (1955) and Lagerlof (1969).

b) Individual motility.

A drop of semen was diluted with few drops of 0.9% Sodium Chloride (Physiological saline) or 3% Sodium Citrate Dihydrate ($Na_3C_6H_5O_7 2H_2O$), and covered with a standard cover slip. Then the percentage of forward progressing motile spermatozoa was estimates to the nearest 5 per cent, after the method of Milovanov et al. (1964) and Lagerlof (1969).

ii) Spermatozoal concentration

The concentration was estimated by using an EEL absorptiometer, with neutral density filter, (Willett and Buckner, 1951), calibrated by careful spermatozoa counts, using Neubauer Cristalite Haemocytometer Slide (Walton, 1927 and Brady and Gildow, 1939).

Raw semen was diluted 1 in 200,using a 0.9% Sodium Chloride Solution (Physiological Saline Solution) to which was added a few drops of detergent (Teepol) to immobilize and reduce agglutination

of the spermatozoa. Correlation of the two methods and the regression equation for the calculation of sperm density from the absorptiometer readings is presented in figure 5 from 14 pairs of observations in table 1.

iii) Spermatozoal morphology

Smears were prepared from the raw and from the diluted semen samples during the different stages of the deep freezing process, just after dilution, at the end of equilibration, and on thawing after 24 hours and one month of storage of the frozen semen.

To study the differential count (live/dead%), and the general morphology of the sperm head, neck and tail the smears were stained with eosin nigrosin stain according to Swanson, and Bearden (1951), and for the morphology of the sperm acrosome smears were stained with eosin fast-green FCF stain, according to Wells and Awa (1970a).

a) Eosin-nigrosin stain

The stain was prepared by dissolving 1g.of the water soluble eosin B (Gurr.) and 5g. of the water soluble nigrosin (Gurr.) in 100ml of sterilised 3% sodium citrate (Dihydrate), and stored in the refrigerator.

One drop of semen (raw or diluted) was mixed with 5-8 drops of the stain and allowed to react for 30 seconds. Care was taken to have all reagents and glass-ware at a constant 37°C. After the staining period a thin smear was made by placing a small drop from the mixture by means of a pasteur pipette on a clean slide, smearing with second slide and drying at room temperature.

For photographical purposes, some of the slides were mounted using Depex mounting medium (Gurr.) and microscopical glass cover-slip (No. 1.5-22x40mm). The slides were examined under the light microscope with oil immersion lens at magnification X 1500. Two hundred spermatozoa were counted, chosen at random from each slide (Campbell, et al., 1957), and classified as dead when the spermatozoa stained with eosin completely red, and live when they stayed unstained or partially stained, figure 11.

b) Eosin and fast green FCF stain

The stain was prepared by mixing one volume of 1% water soluble eosin B (Gurr.) solution, two volumes of 1% water soluble fast green FCF, (Gurr.) solution and 1.7 volume of ethyl alcohol.

For photographical purposes, occassionally the FCF was increased from two to four volumes in order to improve background contrast (figure 25 & 27).

One drop of semen (raw or diluted) was mixed with 5-10 drops of sodium citrate (dihydrate) in a watch glass. One drop of the sperm cell suspension was mixed with one drop of the prepared stain and allowed to react for one minute. One small drop of this mixture was placed on a clean slide, smeared with a second slide, and dried at room temperature. Care was taken to have all reagents and glass-ware at a constant 37^{o}C. Examination of the slides under the light microscope was performed as for nigrosin-eosin smears.

The results of semen evaluations of each sample were recorded on a separate standard form, as illustrated in Appendix 2.

3. DEEP FREEZING PROCEDURE

a) Dilution

The basic diluent components were glycerol, dimethyl-sulphoxide, (DMSO), egg-yolk, lactose, sodium citrate and distilled water.

i) Glycerol 98% $C_3H_5(OH)_3$

ii) Dimethyl-sulphoxide CH_3SOCH_3

iii) Egg-yolk

The egg yolk was obtained from fresh eggs of the domestic fowl (Gallus domesticus). The egg shells were sterilized by washing with water followed by swabbing with cotton moistened with 70% alcohol and drying.

The egg shell was broken in two, using a clean sterilized surgical knife. The whole albumin was poured off by transferring the egg yolk enveloped in its vitelline membrane from one half to another and then on to a filter paper, until the outer surface dried. The vitelline membrane was ruptured by finger pressure through the filter paper and the egg yolk, fraction (free of albumin) poured off into a clean sterile 25ml. beaker.

iv) Lactose

The 11% lactose solution was prepared by dissolving 110g. lactose in 1000ml. distilled water in a 2 litres Erlinmyer flask. Bottles of 100ml capacity were filled with 11% lactose solution, and sterilized by steam for 30 minutes on 3 successive days.

v) Sodium citrate

When a buffer was required, 3g. Sodium citrate dihydrate $Na3C_6H_5O_72H_2O$, were dissolved in 100ml. distilled water. Then equal

volumes of the 3% sodium citrate and of the 11% lactose solutions were used.

All glass-ware was sterilized in a hot air oven at 90-100°C. The whole diluent mixture was emulsified well with a clean sterilized glass emulsifier.

The diluents were usually prepared either on the day of collection or a day before.

Neutralit sticks pH 5-10 were used to measure the PH. of the raw as well as the diluted semen and the diluent. A PH. meter was tried, but proved difficult to use with raw semen. The small volume of the semen ejaculate made it difficult or impossible to immerse fully the bulbs of the PH. meter electrodes. With diluted semen as well as the diluent, the egg yolk particles stuck to the bulbs and obliterated their opening. In addition the sticks were considered sufficiently accurate to ensure that diluents were approximately neutral.

Addition of the diluent to the semen was either directly at once, at 37-39°C (water bath), or by dropping through a fine 40/11 B.D. Yale Microlance Needle, and 5 ml. Everett sterile syringe against the wall of the tubes containing the raw semen, (figure 6). The dropping of the diluents was either at 20°C or 4°C.

The proportion of the ingredients of the diluents and the dilution rates was varied according to experimental requirements,

b) Equilibration

Equilibration time is the period between dilutinn of the raw semen and freezing of the diluted semen on the dry ice. The usual equilibration temperature was 4°C, the tubes containing the diluted semen

being held in an electric refrigerator. In some cases the equilibration temperature was either at room temperature (20°C) or partly at 20°C and partly at 4°C.

c) Freezing and storage

Freezing of the semen to -79°C on dry ice (Carbon dioxide) by the pellet method according to Nagase, and Niwa (1963a) and Fraser (1968) was adopted.

Regular small holes were made on the level surface of a dry ice block by pins fixed on a wooden board (figure 7). The equilibrated semen was dropped into the holes by means of a pasteur pipette. When the droplets solidified the resultant pellets were transferred to a small polythene vials hung on an aluminium carrier which was plunged into Liquid Nitrogen (-196°C) in a Union Carbide L 10 refrigerator cylinder for further storage (figure 8).

d) Thawing

i) Sampling

For each frozen semen sample 2-4 frozen pellets were dropped from the specific vial into a test tube. The whole vial carrier had to be removed from the cylinder containing the liquid nitrogen, especially when the required vial was situated at the lower end (figure 8).

ii) Thawing temperature

Thawing temperature differed according to the experimental requirement. They ranged through 0°C (ice), 20°C, 37°C, 39°C,

45°C, 60°C, 80°C, to 100°C. (boiling water). For thawing of frozen pellets at 37-39°C, the sample tubes were placed in a water bath. At lower and higher temperatures plastic and glass beakers of water were used respectively. The tubes containing the samples were plunged into the beakers and thawing was judged to have occurred at the point of complete melting of the frozen semen pellets, at which point the tubes were transferred to the water bath.

iii) Thawing medium

In most cases the frozen pellets were thawed directly without adding any thawing solution, but in some experiments one of the following thawing media was added to the frozen pellets.

1. Sodium Chloride solution 0.9% at 37°C.
2. Frozen pellets of sodium chloride 0.9% at -196°C.
3. Sodium citrate solution 3% at 37°C.
4. Frozen pellets of sodium citrate 3% at -196°C.
5. Equal volumes of sodium citrate solution 3% and Lactose solution 11% at 37°C.
6. Frozen pellets of equal volumes of sodium citrate 3% and Lactose 11% at -196°C.

The rate of dilution was 2 frozen semen pellets to 3 parts of thawing medium.

4. MATERIAL SPECIFICATION

a) Glycerol $C_3H_5(OH)_3$

Produced by The British Drug Houses Ltd.,
B.D.H. Laboratory Chemical Division,
Poole, England, U.K.

b) Dimethyl-Sulphoxide (DMSO) CH_3SOCH_3

Produced by The British Drug Houses Ltd.,
B.D.H. Laboratory Chemical Division,
Poole, England, U.K.

c) Lactose (A special selected and tested sugar for use in microbiological culture)

Produced by Oxoid Division of Oxo Ltd.,
London, S.W.1.
England, U.K.

d) Sodium Citrate $Na_3C_6H_5O_7 2H_2O$

Produced by The British Drug Houses Ltd.,
B.D.H. Laboratory Chemical Division,
Poole, England, U.K.

e) Sodium Chloride Solution 0.9% W/V (physiological saline)

Produced by Baxter Laboratories Ltd.,
Thetford, Norfolk,
England, U.K.

f) Neutralit PH 5-10

Produced by E. Merck, Darmstadt, Germany.

g) Stains

i - Eosin B. - Water soluble (Gurr.)

ii - Nigrosin - Water soluble (Gurr.)

iii - Fast green FCF - Water soluble (Gurr.)

Produced by George T. Gurr Ltd.,

London, S.W.6,

England, U.K.

h) Absorptionmeter (EEL)

Produced by Evans Electro-Selenium Ltd.,

Halstead, Essex,

England, U.K.

i) Plectron Electro-Ejaculator

Produced by Plectron Corporation, Overton, Nebraska.

Supplied by Alfred Cox (Surgical) Ltd.,

Coulson, Surrey, England, U.K.

j) Lubricating K-Y Jelly- water soluble

Produced by Johnson and Johnson Ltd.,

Slough, England, U.K.

k) Liquid nitrogen container (Union Carbide Cylinder)

Produced by Union Carbide U.K. Ltd.,

London, England, U.K.

5. STATISTICAL ANALYSIS

a) Analysis of Variance

This section illustrates the general methods of analysis for 3 factors experiment according to Snedecor, and Cochran, (1974). The data are taken from the motility percentages of the diluted ram semen, with different glycerol percentages (2, 4, 6 and 8) throughout the different stages of the freezing process (D, E, T_1 and T_2) in the first experiment.

The factors are, samples (6 levels), glycerol percentages (4 levels), and stages of the deep freezing process (4 levels). This makes a 6x4x4 factorial arrangement of treatments in which the samples is random effect, and the other two are fixed effects. From the raw data (Table A9 in the appendix) the grand sum of squares (Σx^2) = 98850, the grand sum (Σx) = 2410, and the correction factor $(\Sigma x)^2/N$ = 60501.041 of 96 observations are computed. The sums of the motility percentages for each factor are listed in tables 2 to 4.

The sum of squares for the separate factors are computed and the analysis of variance set out as illustrated in table 5.

b) Standard errors of difference between means (S.E.D.)

To obtain the standard errors for the factorial experiment the factors are arranged as follows.

Random factor

Sample = A number of levels, a = 6

Fixed factors.

(i) Stages of the freezing process = B number of levels b = 4, mean square for residual in AxB stratum = M1 = 519.86.

(ii) Treatment (Glycerol %) = C; number of levels c = 4, mean squares for residual in AxC stratum = M2 = 90.42.

(iii) The interaction of stages x treatment = BxC; with number of levels = bxc. Mean square for residual in AxBxC stratum = M3 = 36.85.

Then the S.E.D's for comparing the means of the fixed factors (B+C)

i - with different levels of arrangements

$$\text{SED for B} = \sqrt{\frac{M1x2}{a \times c}} = \sqrt{\frac{519.86x2}{6x4}} = 6.582$$

$$\text{SED for C} = \sqrt{\frac{M2x2}{a \times b}} = \sqrt{\frac{90.42x2}{6x4}} = 2.745$$

ii - with same levels of arrangements

$$\text{SED for B at the same level of C} = \sqrt{\frac{M1x2}{a \times c} + \frac{M3x(C-1)x2}{a \times c}} = \sqrt{\frac{519.86x2}{6x4} + \frac{36.45x(4-1)x2}{6x4}}$$

$$= 7.248$$

$$\text{SED for C at the same level of B} = \sqrt{\frac{M2x2}{a \times b} + \frac{M3x(b-1)x2}{a \times b}} = \sqrt{\frac{90.42x2}{6x4} + \frac{36.85x(4-1)x2}{6x4}}$$

$$= 4.092$$

A significant stage effect indicates that the differences in motility % at successive stages are constant throughout the different samples.

For those effects giving significant variance ratios the significant differences, S.D., between a pair of means is calculated from the

formula $SD = \frac{Q \times S.E.D.}{2}$.

where Q is the upper 5% percentage point in the studentized range (Table A15, p568 , Snedecor and Cochran 1974).

Analysis of the data as described was carried out using the GENSTAT package on the E.R.C.C. IBM 360, under the supervision of Dr. R.A. Elton, at the Medical Computing Group, Medical Building, Teviot Place, Edinburgh.

N.B. The counts of the different forms of acrosomal damages viz. swollen, empty, irregular, fractured and multidefective acrosomes, as well as primary defects of the head and neck were low in all experiments and preliminary analysis of variance indicated no significant differences. The acrosome defects were therefore computer analysed as totals only, and analysis of head and neck defects was omitted.

c) T-test

Students t-test as described by Campbell (1974) was used to test the significance of differences between means, where one or both means was derived from data not included in the analysis of variance e.g. to compare the percentages of motile spermatozoa in raw untreated semen with percentage in subsequent stages of the freezing process.

i Paired samples

To find the effect of 2% glycerol dilution on the raw semen motility illustrates the way of calculation (table 6). If the dilution of the raw semen with 2% glycerol is harmless, we would expect the differences (shown in the last column) to be zero or close to it.

Let the difference be called Z. Then $\Sigma Z = -140$, $\Sigma Z^2 = 3800$, and $N = 6$.

So from these six values we can estimate the variance by

$$V = \frac{\Sigma Z^2 - \frac{(\Sigma Z)^2}{N}}{N-1} = 106.67$$

We wish to compare the observed sample mean ($\bar{Z} = -23.33$) with zero which is the mean value stated in the nil hypothesis.

For the t statistics it is convenient to calculate the standard error of the mean as $\sqrt{\frac{V}{N}} = \sqrt{\frac{106.67}{6}} = 4.22$

So t value is $\frac{\bar{Z} - 0}{\sqrt{\frac{V}{N}}} = \frac{23.33}{4.22} = 5.53$ on 5 degrees of freedom.

By referring to the tabulated t values for 5 degrees of freedom, (table A12 pp.357, Campbell 1974), it is clear that the observed value is significant ($P<0.05$).

ii Unpaired samples

The following formula has to be used.

$$t = \frac{\bar{X}1 - \bar{X}2}{\sqrt{\frac{V1}{N1} + \frac{V2}{N2}}}$$

where $\bar{X}$ = mean of the X1 + X2 + XN)

N = number of samples

$$V = \frac{\Sigma X^2 - \frac{(\Sigma X)^2}{N}}{N - 1}$$

1) PRELIMINARY WORK

INTRODUCTION

The inclusion of egg yolk in the diluent is necessary for protection of ram semen against cold shock during cooling and storage at 4°C (Mayer and Lasley, 1944; Bogart and Mayer, 1950; Blackshaw, 1954a and Blackshaw and Salisbury, 1957), as well as during freezing (Jones and Martin, 1965 and Quinn and White, 1966b).

Egg yolk in proportions of 6.5% (Entwistle and Martin, 1972), 15% (Salamon and Lightfoot, 1969), 24% (First et al., 1961a) or 25% (Fraser, 1968) was sufficient to protect ram spermatozoa during storage at 4°C as well as freezing and storage at ultralow temperature.

The presence of non electrolyte in the form of sugars in the diluents improved the survival rate of ram spermatozoa during storage at 4°C and ultralow temperature.

The preferred sugar was lactose according to the findings of Fraser (1962 & 1968); Aamdal and Anderson (1968b); Sainsbury (1968); Loginova and Zeltobruch (1968a); Salamon and Lightfoot (1969); Salamon (1970) and Anderson and Aamdal (1972).

Storage of semen around 5°C can be regarded as an ageing process, which leads to decline in spermatozoal viability (Singh and Sadhu, 1966), and loss of spermatozoal protein (Leslie and Quinlivan, 1968). Equilibration of mammalian spermatozoa for 18-24 hours (overnight) is no more beneficial than 0-0.5 hour and in some cases might be detrimental as claimed by White et al. (1954); Emmens and Martin (1957); Blackshaw et al. (1957); Hill et al. (1959); Bielanski (1973) and Fraser (1973).

Short equilibration of not more than 0.5 hour (Blackshaw et al., 1957 and Hill et al., 1959) or not more than 6 hours (Kuznecov and

Kuprijanova, 1959; Kareta, Pilch and Wierzbowski, 1971 and Fraser, 1973) was indicated for freezing ram semen.

The inclusion of glycerol as a cryoprotective, essential to protect mammalian spermatozoa against injury for freezing has been described in most of the relevant literature since its first introduction by Polge et al (1949) and Smith and Polge (1950 a, b).

The proper glycerol level to provide reasonable protection to ram spermatozoa during freezing was 3.5% (Platov, 1965, 1966) 2.4 or 4% (Lightfoot and Salamon, 1969a), 3.75 (Blackshaw, 1960b) or not more than 4%, higher levels being toxic (Fraser, 1968).

The aim of this preliminary work was to assess the suitability of the provisionally chosen materials and methods and at the same time to study the following:-

A) The effect of storage at $4^{o}C$ on spermatozoa in diluted ram semen.

B) The effect of freezing and storage at $-196^{o}C$ and levels of glycerol in the diluent on ram spermatozoa.

A) EFFECT OF STORAGE AT $4^{o}C$ ON SPERMATOZOA IN DILUTED RAM SEMEN

MATERIALS AND METHODS

After collection and evaluation, the ram semen samples, were diluted with egg yolk, 25%, and lactose (11% solution), 75% at a rate of 1:4 (semen : diluent). Dilution was carried out directly in a water bath at $37^{o}C$.

Following the necessary evaluation, the diluted semen was kept in a refrigerator at $4^{o}C$ for 0, 6.0 and 24.0 hours.

Evaluation of motility was carried out immediately at the end of each storage time.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defects.

Spermatozoa percentage counts in the stained smears were based on counts of 500 spermatozoa per slide. In nigrosin and eosin stained smears, completely unstained spermatozoa were classed as live and partially or completely stained spermatozoa were classed as dead.

General spermatozoal morphology was classified according to the criteria listed in the semen evaluation form (see Appendix 2).

Spermatozoal acrosomal morphology was classified as follows:-

a) Normal acrosome - when the outer limit of the acrosome was smooth and regular.

b) Abnormal acrosome - when the acrosome appeared thick, empty, rough at margin, fractured, showed more than one defect, or was detached partially or completely.

RESULTS

General Observations

On careful physical examination of the genitalia, some rams were found to have preputial injuries and others abnormal hardness of their testicles, and such rams were rejected as sources of semen samples.

The Plectron Electro-ejaculator gave an acceptable performance for semen collection, as judged by the parameters for the raw samples.

The appearance of the raw semen samples was usually associated with density. Dense samples had a thick creamy or yellowish white appearance, and poor samples were thin watery and grey to cloudy in appearance.

Intermediate samples were milky white appearance. Occassionally some ejaculates were tinged with urine or cell debris changing the semen appearance to yellow or brownish respectively.

Experimental Results

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A1 in the appendix. The analyses of variances of the data for the processed semen are presented in table A2 in the appendix.

The mean values of the different parameters in the 4 raw semen samples are presented in table 7. The mean percentages of the different variables in the processed semen samples during the storage period at $4^{o}C$, the standard error of their differences and the significant differences at the 5% level are shown in table 8.

Live Spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 64.25 ± 5.138 to 56.60 ± 3.161. This difference was significant ($P < 0.05$).

Analysis of variance of the spermatozoa live percentages (table A2), shows that there was a significant difference ($P < 0.001$) attributable to storage time at $4^{o}C$.

Storage of the diluted semen samples caused a further and continuous reduction in the spermatozoa live percentages which differed significantly ($P < 0.05$) at 6.0 and 24.0 hours from that at 0 hours.

Motile Spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell from 76.25 $\pm$ 6.884 to 65.0 $\pm$ 9.575. This difference was significant ($P < 0.05$).

Analysis of variance of the spermatozoa motility percentages (table A2), shows that there was a significant difference ($P < 0.05$) attributable to storage time.

Storage caused a further and continuous reduction in the spermatozoa motility percentages. The percentage at 24.0 hours differed significantly ($P < 0.05$) from that at 0 hours.

Spermatozoa with abnormal acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with abnormal acrosomes increased from 21.75 $\pm$ 3.902 to 33.90 $\pm$ 4.022. This difference was significant ($P < 0.05$).

Analysis of variance of the percentages of abnormal acrosomes (table A2), shows that there were no significant differences attributable to storage time, although there was a progressive increase in abnormalities from 0 - 24.0 hours.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoal tail defects increased from 36.85 $\pm$ 6.593 to 39.45 $\pm$ 5.237 but this difference was not significant.

Analysis of variance of the percentages of tail defect (table A2), shows that there was a significant difference ($P < 0.001$) attributable to storage time.

Storage caused a sharp and continuous reduction in the spermatozoa tail defect percentages, which differed significantly ($P < 0.05$) at 6.0 and 24.0 hours from those at 0 hour.

Spermatozoa with head and neck defects

On dilution of the raw semen, the mean percentages of the spermatozoal head and neck defects increased slightly from 1.10 ± 0.580 to 1.50 ± 0.420 but the difference was not significant.

Analysis of variance of the spermatozoal head and neck defect percentages (table A2) shows that there were no significant differences attributable to storage time.

DISCUSSION

Semen characters and other observations

Semen collection by means of the electro-ejaculator from the rams was simple and satisfactory as recommended by many authors since its introduction in the field of A.I. in sheep by Gunn (1936).

The average semen volume was 0.93 ± 0.193ml. which is near to the normal average (ml.) as found by Starke (1949); Lunca (1964); Terrill (1968 & 1969) and Fraser (1971a).

The average individual motility percentage of the raw semen samples was 76.25 ± 6.884 which is near to the figures for normal ram semen as given by Starke (1949); Hulet and Ercanbrack (1962); Lunca (1964); Terrill (1968); Foote (1969) and Foote and Trimberger (1969).

Spermatozoal concentration with average of $147.47 \pm 45.332 \times 10^4/mm^3$ was below the normal concentration of the ram ejaculates. It was lower than the counts of 200×10^4 (Fraser, 1971a) and 250×10^4 (Mattner and Voglmayr, 1962) but was within the range of $138\text{–}178 \times 10^4$ given by

Gunn,Sandners and Granger (1942), Salamon and Morrant (1963) and Visser (1969). In addition some ejaculates were discarded because of the watery consistency of the semen or poor sperm activity, as assessed by both mass and individual motility, which might have been due to the seasonal factor because the collection was done during summer (May - July). Such reduced quality of ram semen during summer was in agreement with the statement of Brady and Gildow (1939); Koger (1951); Asdell (1965) and Mohri et al (1970). The inclusion of the partially stained spermatozoa as a dead led to a big difference between the counts of live and motile spermatozoa, which might be due to regarding the live motile spermatozoa as a dead spermatozoa when taking the stain partially. (Nour Eldin, et al. 1969), or it might be due to the over estimation of the spermatozoal motility or both. It is necessary however to count the partially stained spermatozoa as alive, to reduce this discrepancy.

The drop in spermatozoal activity at the stage of dilution is the result of sudden change in the spermatozoal environment as well as dilution itself being a mechanical stress to the spermatozoa (Polge, 1974).

In addition spermatozoal weaknesses are increased during the period between semen collection and dilution, which can be considered as an in vitro storage at 37°C of the undiluted semen which is quite harmful to the spermatozoa as observed by Willett et al (1940). The drop in the spermatozoa live percentage after dilution is due to either the death of the spermatozoa as the result of these deleterious factors or the eosinophilic count is increased after dilution as a result of increased permeability of the plasma membrane of both dead and immobile live spermatozoa as observed by Dott and Walton (1960) and Eliasson and Treichal (1971).

The sudden drop in spermatozoa motility percentage after dilution has also been observed by Blackshaw (1953); Dott and Walton (1960) and Jones and Holt (1974).

Some samples had to be rejected for further processing because the spermatozoa became almost completely immotile just after dilution which was probably partially due to the poor quality of the semen produced by the ram out of the normal breeding season.

Storage of the diluted ram semen at $4^{o}C$ is harmful as shown by the continuous drop in the sperm live and motility percentages, during the storage of the diluted semen sample at $4^{o}C$ up to 24.0 hours. Such effects might be due to ageing phenomena as found by Singh and Sadhu (1966) or loss of the seminal protein (Leslie and Quinlivan, 1968) or loss of the DNA content of the spermatozoa (Salisbury et al, 1961 and Blackshaw and Salisbury, 1972). Generally cooling of the diluted semen from $37^{o}C$ to near $0^{o}C$ temperature is necessary in order to store the diluted semen before freezing, and $4^{o}C$ is preferred by most workers.

On the other hand the procedure and the time of cooling the diluted semen has to be considered with care otherwise semen might be subjected to cold shock (Birillo and Puhaljskii, 1936; Gladcinova, 1937; Easley et al, 1942; Lasley and Mayer, 1944; Lasley and Bogart, 1944 and Mann and Lutwak-Mann, 1955).

Cooling of the diluted semen throughout the experimental work was done in an electric refrigerator and the temperature fall to $4^{o}C$ took 30 minutes which is very short in the view of Birillo and Puhaljskii (1936); Mann and Lutwak-Mann(1955) and Walton (1957).

Acrosomal defects started to increase on dilution of the raw semen, which might be due to the mechanical injuries of dilution or the presence of a sticky material at the anterior part of the spermatozoa head as observed

in the hamster and mouse spermatozoa (Phillips, 1972) or the presence of specific antibody in the media after dilution of the spermatozoa of ram and bull (Bedford, 1965c) or of the rabbit (Bedford, 1965c & 1970) which leads to the head agglutination of the spermatozoa.

Dilution of semen also increases the possibility of spermatozoal agglutination as observed in the ram by Dott and Walton (1960) and Bedford (1965c) and in the bull by Lindahl (1968a & 1973). Such agglutination phenomena may predispose to plasma membrane breakage and increase in spermatozoa showing acrosomal defects as observed by Jones (1972b) and Jones and Holt (1974) in ram, Pursel et al. (1970a & 1972a) in boar, and Healey and Weir (1970) in chinchilla semen.

In addition acrosomal damage might be increased after dilution of the raw semen as a result of the cytochemical changes in the spermatozoa as observed by Garner et al. (1971) and Jones and Holt (1974). The increase in the acrosomal abnormalities throughout storage might be due to a continuous process of acrosomal reaction (Bedford, 1969 and Williamson, 1974b), or acrosomal degeneration (Garner et al., 1971).

Acrosomal defects increased on storage at 4°C up to 24.0 hours. which might be due to the sensitivity of the ram spermatozoa to such condition being greater than that of other species (bull) as found by Baicoianu and Dimoftache (1968), or due to the loss of the phospholipid from the spermatozoa on cooling and storage at 4°C (Mukhergee, 1964). In addition spermatozoal activity during storage at such temperature might increase the possibility of acrosomal defects (Schroder, 1964).

Tail defects usually increased after dilution as a reaction to the new environment (Drevius, 1963 & 1972; Drevius and Eriksson, 1966, and Quinn et al., 1968b).

The reduction of tail defects observed on storage might be simply due to reduced spermatozoal activity.

Head and neck defects were very few and their increase on dilution and further storage at 4°C was very slight. These defects might be due to the ageing of the spermatozoa during storage at 4°C. In addition smearing of the semen samples might lead mechanically to secondary head defects.

CONCLUSION

1) Semen collection by the electro ejaculator was satisfactory.

2) Storage of the diluted ram semen at 4°C has to be as short as possible to avoid unnecessary further damage or loss of the viability of the spermatozoa.

3) The discrepancy between live and motile spermatozoa counts is probably due to counting partially stained spermatozoa as dead. However, whether to count them as live will be decided at the end of the preliminary freezing.

B) EFFECT OF FREEZING AND STORAGE AT -196°C AND LEVEL OF GLYCEROL ON RAM SPERMATOZOA

MATERIALS AND METHODS

1) After collection and evaluation, the raw semen samples were divided into four equal volumes in 10ml. tubes, and one of the following diluent was added directly at a dilution rate of 1:4 (semen:diluent). All

dilutions were carried out at 37°C in a water bath.

a) 2% glycerol, 73% lactose (11% solution) and 25% egg yolk.

b) 4% " , 71% " " " " " " "

c) 6% " , 69% " " " " " " "

d) 8% " , 67% " " " " " " "

These dilution rates resulted in final glycerol percentages of 1.6, 3.2, 4.8 and 6.4 respectively.

2) The diluted semen samples were equilibrated at 4°C for 2 hours, followed by freezing on dry ice (-79°C) and storage in liquid nitrogen (-196°C).

3) Thawing of the frozen pellets was done in a dry test tube in hot water at 60°C after 24 hours, and one month.

4) Eosin nigrosin and eosin fast green FCF stained smears were prepared immediately after dilution, at the end of 2 hours equilibration, and following thawing after ultra low temperature storage at (-196°C) for 24 hours and one month, for subsequent evaluation of percentage of live spermatozoa and morphological defects.

5) Spermatozoal percentage counts in the stained smears were based on counting 200 spermatozoa per slide. Completely unstained spermatozoa were classed as live and completely or partially stained spermatozoa as dead.

6) The spermatozoal acrosomal morphology was classified as follows:-

a) Normal acrosome - when the outer line of the acrosome was smooth and regular.

b) Damaged acrosome - when the acrosome presented one of the following features:

i) Swollen acrosome - The acrosome became thicker and larger than normal.

ii) Empty acrosome - The acrosome showed a large vacoule at the anterior part.

iii) Irregular acrosome - the outer layer became thick and not smooth.

iv) Fractured acrosome - The outer layer became fissured or part of the acrosome sloughed away.

c) Detached acrosome - when the acrosome was partially detached or missing.

N.B. When the acrosome showed more than one abnormality, it was classified according to the most obvious one.

d) Acrosomal defects - this term is used to denote the sum of damaged and detached acrosomes as defined.

7) Five replicates were evaluated for each level of glycerol.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentage in table A3 in the appendix.

The analysis of variance of the data for the processed semen are presented in tables A4 - A8 in the appendix.

The mean values of the different parameters in the raw semen samples are presented in table 9.

The mean percentages of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% level are shown in table 10 - live, 11 - acrosomal damage, 12 - acrosomal detachment, 13 - acrosomal defects and 14 - tail

defects. The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 9 and as affected by level in figure 10.

Live Spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 61.80 $\pm$ 1.800 to 50.80 $\pm$ 3.088. There were significant differences ($P < 0.05$) between the counts for raw semen and semen diluted with 6 or 8% glycerol.

Analysis of variance of the percentages of live spermatozoa (table A4) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) Equilibration of the diluted semen samples caused a further reduction in live percentage and which differed significantly ($P < 0.05$) from that at the stage of dilution.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month caused a further fall in live percentage. The live percentage in samples thawed after 24 hours and one month differed significantly ($P < 0.05$) from those at all the previous stages.

2) The highest live percentage was found in samples diluted with 2% glycerol followed by 4, 6 and 8%. Live percentage in samples diluted with 2% glycerol differed significantly ($P < 0.05$) from those in samples

diluted with 6 and 8%. Live percentage in samples diluted with 4% glycerol differed significantly ($P < 0.05$) from those in samples diluted with 8%.

3. Following all four stages of the freezing process, live percentage decreased as the glycerol level increased from 2 to 8% glycerol. Live percentage in samples diluted with 2% glycerol differed significantly ($P < 0.05$) from those in samples diluted with 8% glycerol throughout the different stages of the freezing process, as well as from those in samples diluted with 6% glycerol following dilution and equilibration. Live percentage in samples diluted with 4% glycerol differed significantly ($P < 0.05$) from that in samples diluted with 8% glycerol following equilibration only.

The fall in live percentage from dilution to equilibration was least in samples diluted with 4% glycerol. The largest fall was in samples diluted with 8% glycerol followed by 2 and 6% and these three differences were all significant ($P < 0.05$).

The further fall in live percentage from equilibration to freezing and thawing was significant in 2 and 4% glycerol when thawed after 24 hours and in 2, 4 and 6% glycerol when thawed after one month.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of damaged acrosomes decreased from 12.60 ± 1.756 to 7.50 ± 1.547. There was a significant difference ($P < 0.05$) between the counts for raw semen and semen diluted with 4% glycerol.

Analysis of variance of the percentages of spermatozoa with damaged acrosomes (table A5) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal detachments increased from 3.20 ± 1.007 to 14.67 ± 0.769. There was a significant difference ($P < 0.05$) between the counts for raw semen and semen diluted with 2, 6 or 8% glycerol.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A6) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) Equilibration of the diluted semen samples caused a further increase in the acrosomal detachment percentage, which differed significantly ($P < 0.05$) from that at the stage of dilution.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month also caused a further increase in the percentages of detached acrosomes which differed significantly ($P < 0.05$), from those at the stages of dilution and equilibration.

2) Acrosomal detachment percentage was highest in samples diluted with 2% glycerol followed by 6, 8 and 4, but the differences were small.

3) Following the different stages of the freezing process, also, acrosomal detachment percentages differed very slightly between the glycerol levels.

The increase in acrosomal detachment from dilution to equilibration was greatest in samples diluted with 6% glycerol followed by 4, 2 and 8%. The further increase in acrosomal detachment percentage from equilibration to thawing was nearly the same in all glycerol levels but greatest in samples diluted with 8% glycerol.

Total spermatozoa with acrosomal defects (i.e. damaged plus detached)

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased from 15.80 ± 2.065 to 22.17 ± 1.791. There was a significant difference ($P < 0.05$) between the count for raw and semen diluted with 8% glycerol.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A7) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) Equilibration of the diluted semen samples caused a further but not significant increase in acrosomal defects.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused further increases in the percentages which differed significantly ($P < 0.05$) from that following dilution.

2) The highest percentage of acrosomal defects was found in samples diluted with 2% followed by 8, 6 and 4% glycerol but the range was small.

3) Following several stages of the freezing process acrosomal defect percentages differed only slightly between glycerol levels. However, the highest percentage of defects was found in samples diluted with 8 and 2% glycerol followed by 4 and 6% following both dilution and thawing after 24 hours, with 4 and 2 followed by 8 and 6% following equilibration, and with 6 followed by 2, 8 and 4% following thawing after one month.

The greatest increase in acrosomal defects from dilution to equilibration was found in samples diluted with 4% glycerol followed by 6, 2 and 8%.

The further increase in acrosomal defect percentage from equilibration to thawing was greatest in samples diluted with 8% glycerol followed by 6, 2 and 4%, when thawed after 24 hours but with 6, followed by 8, 4 and 2% when thawed after one month.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoal tail defects increased slightly from 25.60 ± 4.205 to 31.55 ± 1.733. The largest, but not significant increase occurred in samples diluted with 8% glycerol followed by 4, 2 and 6%.

Analysis of variance of the percentage of spermatozoa with tail defects (table A8) shows the following:-

1) There was a significant difference ($P < 0.001$) contributable to stage of the freezing process.

2) There was no significant difference contributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) Equilibration of the diluted semen samples caused a marked reduction in tail defect percentage, which differed significantly ($P < 0.05$) from that following dilution.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month, caused a further reduction in tail defect percentages which differed significantly ($P < 0.05$) from that in samples following dilution but not equilibration.

2) The percentage of tail defects was lowest overall in samples diluted with 4% glycerol followed by 2, 6 and 8%.

3) Following dilution and equilibration, the differences in tail defect percentages were very small between glycerol levels.

Following thawing, however, the percentage was lowest in samples diluted with 4% glycerol followed by 8, 6 and 2 when thawed after 24 hours and by 8, 2 and 6 when thawed after one month, but none of the differences was significant.

The reduction in tail defect percentage was greatest from dilution to equilibration, the difference being significant ($P < 0.05$) in all glycerol levels.

The reduction in spermatozoal tail defects percentages from equilibration to thawing was not significant, but the largest reduction

was found in samples diluted with 8% glycerol followed by 4, 2 and 6 when thawed after 24 hours and with 4 and 8 followed by 2 and 6 when thawed after one month.

Figure 9 shows that over the whole freezing process the drop in live percentages was associated with an increase in acrosomal damage and reduction in tail defects.

Figure 10 shows that spermatozoa live percentage decreased as the glycerol increased from 2 to 8% but morphological defects (acrosomes and tails) were least with 4%.

DISCUSSION

Analysis of variance of the different variables of the processed semen throughout the stages of the freezing process showed significant differences ($P < 0.05$ and $P < 0.001$) between the replicates which indicate that the freezability differences exist between ejaculates of the same ram (Cheviot C18) and this is in agreement to the observations of White et al (1954); O'Dell and Hurst (1956); Hill et al (1959); Sainsbury (1968); Salamon (1968); Tsakalof, Koutsouris, Samouelidis, Margaritis and Mikas (1974) and Beck and Silverstein (1975). At the end of each stage of the freezing process there was a reduction in the spermatozoal viability (live percentages) associated with acrosomal defects. However, the greatest changes occurred after the actual freezing and storage especially when thawed after one month.

Ram semen has been found to be more sensitive to freezing than buck semen (Tsakalof et al, 1974) and bull semen (Hill et al, 1959; Healey, 1969 and Watson and Martin, 1972). Spermatozoa of the bull

(Mann and Lutwak-Mann, 1955 and Mann, 1969), and of man (Jahnel, 1938) but not of the ram (Salamon, 1968 & 1970) withstand freezing as rapid as dropping directly into liquid nitrogen. Moreover semen collected by electro ejaculator mostly came from the ampullae, i.e. the first part of the ejaculate which contains about half the amount of the phospholipid of the cauda epipidymis, i.e. the last part of the ejaculate (Lasley et al, 1942; Pickett and Komarck, 1967 and Poulos, Brown, Cox and White, 1974) which in turn lowers the spermatozoal resistance to cold shock during cooling and freezing (Larson and Graham, 1958; White and Wales, 1960; Polge and Butler, 1973; Butler and Roberts, 1975 and Coulter and Foote, 1975).

The presence of extra seminal plasma as is the case with electro ejaculator on its addition to the semen provokes acrosomal damage and freezing damage (Hibbitt and Moore, 1975) and also increases the susceptability of the spermatozoa to cold shock during cooling and freezing (Choong and Wales, 1962; Moule, 1970 and Pursel et al, 1972a).

Damage also extended to the tail especially the midpiece, associated with damaged mitochondria (Quinn et al, 1969 and Nath, 1972). Such damage leads to severe reduction in the spermatozoal activity following the breakdown of the Adenotriphosphate (ATP) as observed by Mann and Lutwak-Mann (1955) and other phospholipids as observed by Quinn (1968); Quinn and White (1968); Quinn et al (1969) and Darin-Bennett et al (1973 & 1974). Other chemical substances such as cation and enzymes are also released from the spermatozoa following the different stages of the freezing process as observed by Blackshaw and Salisbury (1957); Nath (1972) and Church, Graves and Macleod (1975).

The transportation of the diluted semen from 4°C to the dry ice by the pasture pipette also may lead to exposure of the spermatozoa to air temperature as observed by Visser (1974a) which in turn increases the morphological defects.

The harmful effect of freezing and thawing of the spermatozoa might be the combination of both intra cellular (Smith and Polge, 1950a) and extra cellular (Watson and Martin, 1974 & 1975) ice crystallization. However, the presence of the glycerol appeared to reduce the mechanical damage by altering the physical and chemical states of the electrolyte and non electrolytes after they had been concentrated by freezing.

According to Salamon (1970) the use of dry ice for very rapid freezing (pelleting of the semen) has the disadvantages that dry ice disintegrates after repeated immersion into liquid nitrogen for cooling and further storage, therefore it can be expected to be an additional factor affecting the survival and the soundness of the ram spermatozoa during freezing and storage.

In respect to the pellet volume, it was kept constant as possible throughout this experiment, but in any case, Nagas and Niwa (1964) and Lightfoot and Salamon (1969b) found no difference between the different pellet volumes and the post-thawing survival of the frozen spermatozoa.

The ultra low temperature storage of the frozen ram semen caused a severe reduction in the percentage of live spermatozoa as well as a reduction in tail defects and an increase in acrosomal defects especially after one month's storage. Reduction in the spermatozoal viability has been observed by Hill et al (1959); First et al (1961a); Sainsbury (1968); Patt and Nath (1969) and Mielikovic, Naumov, Atanasov, Mihailovski, Mrvos, Tanev, Stojadinovic and Stojanivic (1974)in ram semen and by

O'Dell and Hurst (1956); Van Demark, Miller, Kinney, Rodriques and Riedman (1957); Larson and Graham (1958) ; Martin and Emmens (1958) and Martin (1965b) in bull semen stored in liquid nitrogen for various periods from 2 days to several months. On the contrary Polge et al (1949) ; Polge and Rowson (1952b); Szumowski et al (1956) and Flechon (1974) found no change after 4 weeks storage at -79°C of ram, bull, or rabbit semen.

The sharp increase in the eosinophilic count of the spermatozoa during the freezing process especially after freezing followed by thawing might be due to the continuous destructive damage leading to death, which takes place in the plasma and acrosomal membranes as well as the post nuclear cap (as illustrated in plates 59-69) and facilitates the passage of the eosin stain (Dott and Walton, 1960). However, in addition the actual freezing process might increase the passage of the eosin stain in the live spermatozoa through the pores which are already present in the post nuclear cap of the ram spermatozoa (Wu and McKenzie, 1955 and Dott, 1969) or the bulge of the nuclear membrane (Nath, 1972).

The reduction in the live percentages increased as the glycerol level increased from 2 to 8%. This indicates that there is an increase in the toxicity of the glycerol especially after 4% (Fraser, 1968 and Colas, 1975) or an interaction between glycerol and eosin staining which is in agreement with Bruce (1953); Rowson (1953); Martin and Emmens (1958); Martin (1963 a, d, & e and 1965a); Rathore (1965); Jones and Martin (1965) Jones (1965b & 1969b) and Turvey and O'Hagan (1972).

Moreover the claim of Nour Eldin et al (1969) could be added which includes that some spermatozoa stain with eosin while still alive but either immotile or with sluggish motility.

Acrosomal defects such as acrosomal damage and acrosomal detachment during the different stages of the freezing process seemed to vary with severity of the treatment. They increased after dilution, followed by further increase after storage at 4°C (equilibration) of the diluted semen, and the greatest increase after freezing followed by thawing (figure 9) irrespective of glycerol levels. Such variation in the severity of the acrosomal defects is in agreement with the observation of Walton (1957); Hill et al (1959); Quinn et al (1968 a & b); Healey (1969); Wells and Awa (1970a); Graham et al (1971); Nath (1972); Jones (1972a); Jones and Martin (1973); Srivastava et al (1974); Watson and Martin (1974); Hibbit and Moore (1975)and Watson (1975 a & b).

CONCLUSION

1) There were differences in the effects of freezing between samples of the same ram, (Cheviot C18).

2) There was a continuous reduction in the spermatozoal activity (live percentages) associated with an increase in the acrosomal damages following each stage of the freezing process. However the greatest changes occurred after freezing followed by thawing after one month's storage in liquid nitrogen.

On the other hand tail defects showed a slight reduction following each stage of the freezing process.

3) Live spermatozoa decreased as the glycerol level increased from 2 to 8%. On the other hand abnormal spermatozoal morphology was least with 4% glycerol. However the viability was judged by the eosinophilic count only which might have been affected by glycerol interaction with stain so no definite conclusion could be drawn at this stage. There was

also the possibility of an increased susceptibility to freezing of semen collected in the non breeding season (June - July) of the sheep.

4) The drop in live percentages was usually associated with an increase in the morphological defects of the acrosome and reduction of the tail defects. These changes were more affected by freezing stages than by glycerol level.

5) Head and neck defects were affected very slightly by the stages only and such small variation does not justify analysis of variance.

6) It will be necessary in further experimental work to modify methods as follows,

a) Inclusion of assessment of motility in evaluation of the raw as well as the processed semen samples.

b) Classification of completely stained spermatozoa in nigrosin eosin smears as dead and partially stained and unstained spermatozoa as live. The inclusion of partially stained spermatozoa in this preliminary study dead category undercovered the live percentage compared with motility percentages and there is also evidence that glycerol increases the tendency of some live spermatozoa to take up eosin stain.

c) The classification of acrosomal abnormalities adopted gave rise to difficulty when acrosomes showed more than one defect. Such acrosomes are more simply classified separately as multi-defective, unless classified as detached.

2) COMPARISON OF COUNTS OF 200 and 500 SPERMATOZOA PER SLIDE

INTRODUCTION

For the microscopical studies of the spermatozoa under the light microscope such as live percentage, counts of 500 spermatozoa per slide were necessary according to Lagerlof (1969) and Bane (1969). On the other hand most research workers agree that counts of 200 spermatozoa per slide are sufficient for accuracy. Moreover Watson (1975b) has since claimed that a rapid and repeatable estimate of the state of the acrosomes in a sample could be made from the mean score of 20 spermatozoa examined per slide.

The aim of this work was to determine whether there was a significant difference between percentages based on counts of 200 and 500 spermatozoa per slide.

MATERIALS AND METHODS

16 different smears of ram spermatozoa stained with eosin-nigrosin stain were examined under the light microscope. The percentage of dead spermatozoa, classified on the basis of complete staining, was estimated after counting 200 and 500 spermatozoa in each smear.

RESULTS

The result of estimates of the percentage of dead spermatozoa based on counts of 200 and 500 spermatozoa per smear in 16 samples is presented in table 15. The t-test shows that the difference between the pairs of counts was not significant. The maximum discrepancy observed

for any pair of counts was 5.0%.

In addition, the Chi-square test showed that there were no significant differences between the results obtained by counting the first 200 spermatozoa and the next 300 spermatozoa, except in slide 6 where the counts for dead spermatozoa were 21.0 and 29.3% respectively. ($X^2 = 3.91$ D.F=1).

DISCUSSION

The results showed that the differences between estimates based on counts of 200 and 500 spermatozoa per slide were small and not significant. Therefore the count of 200, as generally adopted by most workers, is sufficiently accurate, and the much more time consuming count of 500 is not justified.

CONCLUSION

Percentages will be based on counts of 200 spermatozoa per slide in the following experimental work.

3) SPERMATOZOAL MORPHOLOGY

The following classification of spermatozoal morphology was adopted following the preliminary studies. The general as well as the abnormal morphology of the ram spermatozoa in the raw and the processed semen samples was photomicrographed and is illustrated in figures 11 to 28 (X 2275).

General morphology

The general spermatozoal morphology was evaluated in smears stained with eosin and nigrosin (Swanson and Bearden, 1951) and classified as follows:-

I - Normal spermatozoa

1) Live - partially or completely unstained ... figure 11

2) Dead - completely stained ... figure 11

II - Abnormal spermatozoa

1) Primary defects of the head

a) Pyriform head ... figure 11

b) Dwarf head ... figures 13 14 & 19

2) Primary defects of the tail

a) Proximal cytoplasmic droplet ... figure 12

b) Distal cytoplasmic droplet ... figure 14

c) Thick midpiece ... figures 11 & 12

d) Double midpiece ... figure 11

e) Abaxial attachment ... figures 13, 15, 17 & 19

3) Secondary defects of the head and neck

a) Free head ... figure 16

b) Fractured head ... figure 16

c) Broken neck ... figure 12

4) Secondary defects of the tail

a) Bent tail ... figure 17

b) Looped tail ... figure 19

c) Terminally coiled tail ... figure 15

d) Tail coiled under the head ... figures 13 & 19

e) Tail coiled over the head ... figure 18

f) Broken tail ... figure 14

Acrosomal morphology

The spermatozoal acrosomal morphology was evaluated in smears stained with eosin fast green FCF (Wells and Awa, 1970a) and classified as follows:-

I - Normal acrosome

The acrosome is smooth and closely covers the nucleus figure 22 & 27

II - Abnormal acrosome

1) Swollen - the acrosome is thicker than normal figure 20,25 & 27

2) Empty - the acrosomal content has disappeared and the acrosome has lost its density ... figure 20 & 21

3) Irregular - the acrosome is rough or corrugated especially at the margins ... figure 21 & 24

4) Fractured - the outer layer of the acrosome is fractured ... figure 26

5) Multi-defective - the acrosome shows more than one of the above abnormalities ... figure 21 & 23

6) Detached - the acrosome has sloughed from the spermatozoa head leaving part of it in contact with the anterior margin of the head or has disappeared completely leaving the nucleus denuded ... figure 24 to 28

4) EXPERIMENT NO. 1

THE EFFECT OF GLYCEROL ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

It has been generally accepted that freezing of the living tissue is not possible unless glycerol (Polge et al, 1949 and Smith and Polge, 1950 a & b) or other cryoprotective is included in the diluent. But the optimum level of the glycerol has to be used otherwise it is toxic to the spermatozoa when the glycerol percent exceeds 4% as found in the preliminary freezing experiment which is similar to the findings of Fraser (1968 & 1970).

The optimum glycerol level for freezing ram semen varied in different investigations, from as low as 2% (Salamon, 1970) to as high as 7.5% (Jones, 1965b) or 8% but not more than 10% (First et al, 1961a). In addition it is well known that the different stages of the freezing process are an environmental stress to the spermatozoa and the severity varies according to the treatment.

The aim of this experiment was to study the effect of various glycerol percentages in the diluent throughout the different stages of the freezing process, on spermatozoal viability and morphology and to determine the optimum glycerol percentages for use in subsequent experiments. It is essentially a repitition of the preliminary experiment, where the results may have been affected by the poor quality of the available ram semen samples, and did not include an assessment of motility.

MATERIALS AND METHODS

These were exactly as in the preliminary freezing experiment, except as follows:-

1) The diluted semen samples were equilibrated at $4^{o}C$ for one hour.

2) Evaluation of motility was carried out immediately after dilution, at the end of one hour equilibration, and following thawing after ultra low temperature storage ($-196^{o}C$) for 24 hours and one month.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defects.

3) Six replicates were evaluated for each level of glycerol.

4) The partially or completely unstained spermatozoa were classed as live and only completely stained spermatozoa as dead.

5) Inclusion of the multi-defective acrosomal abnormality in the acrosomal assessment.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A9 in the appendix. The analyses of variance of the data for the processed semen are presented in tables A10 to A15 in the appendix.

The mean values of the different parameters in the six raw semen samples are presented in table 16.

The mean percentages of the different variables in the semen samples, the standard errors of their differences and the significant differences at the 5% level are shown in tables 17 - live, 18 - motility, 19 - acrosomal damage, 20 - acrosomal detachment, 21 - acrosomal defects

and 22 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 29 and as affected by glycerol level in figure 30.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 85.83 $\pm$ 2.600 to 79.60 $\pm$ 3.488. There were significant differences ($P < 0.05$) between the counts for raw semen and semen diluted with 6 or 8% glycerol.

Analysis of variance of the percentage of live spermatozoa (table A10) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to glycerol level.

3) There was a significant interaction ($P < 0.001$) between glycerol level and stage.

1) At the end of equilibration of the diluted semen samples there was a further reduction in the live percentage but the differences between the two stages was not significant.

Freezing of the equilibrated samples followed by thawing after 24 hours caused a marked fall in live percentage. The live percentage at this stage differed significantly ($P < 0.05$) from those of the dilution and equilibration stages. The greatest reduction in the live percentage was found on thawing after one month's storage when it differed significantly ($P < 0.05$) from all the previous stages.

2) The highest spermatozoa live percentage was found in samples diluted with 4% glycerol followed by 2, 6 and 8%. There was a significant difference ($P < 0.05$) between 2 and 8% glycerol and between 4 and either 6 and 8% glycerol.

3) Following dilution live percentage decreased as the glycerol percentage increased from 2 to 8%. Following equilibration, live percentage decreased as the glycerol level decreased or increased from 4%. After either of these two stages, however, were there significant differences between means. On thawing after 24 hours and one month's storage, live percentages again decreased as the glycerol percent decreased or increased beyond 4%. There was a significant difference ($P < 0.05$) between either 2 or 4 and 8% glycerol and between 4 and 6% glycerol. The fall in live percentage following each stage of the freezing process was greatest in samples diluted with 8% glycerol followed by 6, 2 and 4%.

The drop in live percentage from dilution to equilibration was not significant in any of the different glycerol levels. The further drop in live percentage from equilibration to thawing was significant ($P < 0.05$) in samples diluted with 6 and 8% glycerol when thawed after 24 hours and with all the different glycerol levels when thawed after one month.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell from 71.67 ± 7.491 to 44.17 ± 4.657. There were significant differences ($P < 0.05$) between the motility percentages of the raw semen and semen diluted with glycerol at all levels. In addition six samples had to be discarded because their spermatozoa became totally

immobile soon after dilution.

Analysis of variance of the percentage of motile spermatozoa (table A11) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to glycerol level.

3) There was a significant interaction ($P < 0.05$) between glycerol level and stage.

1) At the end of equilibration of the diluted semen samples there was a further reduction in the motility percentage, but the difference between the two stages was not significant. Freezing of the equilibrated samples followed by thawing after 24 hours caused a marked drop in motility percentage. At this stage it differed significantly ($P < 0.05$) from that of the dilution stage but not from that of the equilibration stage.

The greatest reduction in the motility percentage was found on thawing after one month's storage when it differed significantly ($P < 0.05$) from those of both the dilution and equilibration stages.

2) The highest spermatozoal motility percentage was found in samples diluted with 4% glycerol, followed by 6, 2 and 8%. There was a significant difference ($P < 0.05$) between 4 and 8% glycerol only.

3) Following the stage of dilution, the motility percentages decreased as the glycerol percent increased from 2 to 8%, but there were no significant differences between means.

Following the stage of equilibration the highest motility percentage was in samples diluted with either 2 or 4% glycerol followed by 6 and 8%. There was a significant difference ($P < 0.05$) between either 2 or 4 and 8% glycerol.

On thawing after 24 hours and one month's storage the highest motility percentage was in samples diluted with 4% glycerol followed by 6, 2 and 8%. There was a significant difference ($P < 0.05$) between 4 and either 2 or 8% glycerol when thawed after 24 hours storage and between 4 and 8% glycerol when thawed after one month's storage.

The drop in motility percentages from the stage of dilution to the stage of equilibration was slight and not significant in all the different glycerol levels but the largest drop was in samples diluted with 8% glycerol followed by 2 and either 4 or 6%.

The further drop in motility percentages from the stage of equilibration to thawing was greatest in samples diluted with 2% glycerol followed by 6, 8 and 4% when thawed after 24 hours storage; and by 6, 4 and 8 when thawed after one month's storage. The difference was significant ($P < 0.05$) only in case of samples with 2% glycerol when thawed after 24 hours storage and with 2 and 6% glycerol when thawed after both 24 hours and one month's storage.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of damaged acrosomes increased from 6.67 ± 3.175 to 10.92 ± 2.221. There were significant differences ($P < 0.05$) between the counts for raw semen and semen diluted with glycerol at all levels.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A12) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was no significant interaction between glycerol levels and stages.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentages of spermatozoa acrosomal detachments increased from 2.08 ± 0.638 to 6.29 ± 1.693. There were significant differences ($P < 0.05$) between the count for raw semen and diluted semen, irrespective of glycerol level.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A13) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) At the end of equilibration of the diluted semen samples there was a further increase in the acrosomal detachment percentage, but the difference between the two stages was not significant.

Freezing of the equilibrated samples followed by thawing after 24 hours also caused a further increase in percentage of acrosomal

detachments, when it differed significantly ($P < 0.05$) from that at the stage of dilution but not from that at the stage of equilibration.

The greatest increase in the percentage of the acrosomal detachment occurred on thawing after one month when it differed significantly ($P < 0.05$) from those at the stages of dilution and equilibration.

2) Acrosomal detachment percentages increased as the glycerol percentage increased from 2 to 8%. There was a significant difference ($P < 0.05$) between 2 and 8% glycerol.

3) Following the stages of dilution and equilibration acrosomal detachment percentages were not significantly affected by the different glycerol levels, but they were least in samples diluted with 6% glycerol after dilution and with 2% glycerol after equilibration.

On freezing followed by thawing after 24 hours and one month's storage, acrosomal detachment percentages increased as the glycerol percent increased from 2 to 8%. There was a significant difference ($P < 0.05$) between 2 and 8% glycerol.

The increase in acrosomal detachment from the stage of dilution to the stage of equilibration was greatest in samples diluted with 6% glycerol, followed by 8, 4 and 2% but there was no significant difference between means.

The further increase in the acrosomal detachment percentages from the stage of equilibration to the stage of freezing followed by thawing after 24 hours storage was greatest in samples diluted with 8% glycerol followed by 6, 2 and 4%. The difference was significant only in case of 8% glycerol. A greater increase in the acrosomal detachment percentages occurred from the stage of equilibration to the stage of freezing followed by thawing after one month's storage. They increased as the

glycerol percentages increased from 2 to 8% and the difference was significant ($P < 0.05$) in all four glycerol levels.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of acrosomal defects increased from 8.75 ± 3.100 to 17.21 ± 1.647. There were significant differences ($P < 0.05$) between the counts for raw semen and diluted semen irrespective of glycerol level.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A14) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was a significant interaction ($P < 0.05$) between glycerol levels and stage.

1) At the end of equilibration of the diluted semen samples there was a further increase in acrosomal defects, but the differences between the two stages was not significant.

Freezing of the equilibrated samples followed by thawing after 24 hours caused a marked increase in the percentage of acrosomal defects when it differed significantly ($P < 0.05$) from those of the equilibration and dilution stages.

The greatest increase in acrosomal defect percentage was found on thawing after one month, when it differed significantly ($P < 0.05$) from those of all the previous stages.

2) The acrosomal defect percentages increased slightly overall as the glycerol per cent increased from 2 to 8%.

3) Following dilution and equilibration acrosomal defect percentages were not significantly affected by the different glycerol levels but they were least in samples diluted with 6% glycerol.

On thawing, acrosomal defect percentages increased as the glycerol level in the diluent increased from 2 to 6% when thawed after 24 hours and from 2 to 8% glycerol when thawed after one month. There was a significant difference ($P < 0.05$) between 2 and 6% when thawed after 24 hours and between 2 and 8% glycerol when thawed after one month. The increase in acrosomal defects from dilution to equilibration was not significant. The largest increase was with either 4 or 6% glycerol followed by 8 and 2%.

The further increase in acrosomal defect percentages from equilibration to thawing after 24 hour stage was greatest in samples diluted with 6% glycerol followed by 8, 4 and 2%. The differences were significant ($P < 0.05$) in case of 4, 6 and 8% glycerol. Acrosomal defect percentages increased from equilibration to thawing after one month's storage as the glycerol percentages increased from 2 to 8%. The differences were significant ($P < 0.05$) in all glycerol levels.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoa tail defects fell slightly, from 30.17 ± 6.251 to 24.35 ± 3.343. The difference was not significant.

Analysis of variance of the percentage of spermatozoa with tail defects (table A15) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to glycerol level.

3) There was no significant interaction between glycerol level and stage.

1) At the end of equilibration of the diluted semen samples there was a further reduction in the spermatozoa tail defect percentage, but the differences between the two stages was not significant.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month caused the greatest reduction in the spermatozoa tail defect percentages. The tail defect percentages at the thawing stages differed significantly ($P < 0.05$) from those of the equilibration and dilution stages.

2) The lowest tail defect percentage was found in samples diluted with 6% glycerol followed by 4, 2 and 8%, but the range was small.

3) Variations due to glycerol levels at the different stages were also small, the range being 19.83-27.33%, 12.17-23.00%, 1.83-3.83% and 1.42-5.92% following the stages of dilution, equilibration, thawing after 24 hours, and thawing after one month respectively.

The reduction in tail defect percentages from dilution to equilibration was not significant but was largest in samples diluted with 4% glycerol followed by 2, 6 and 8%.

The further reduction in tail defect percentages from equilibration to thawing was greatest in samples diluted with 8% glycerol followed by 2, 6 and 4% when thawed after both 24 hours and one month's storage. The difference was significant ($P < 0.05$) only in case of 8% glycerol after 24 hours storage.

Figure 29 shows that there was a decrease in the percentage of live and motile spermatozoa throughout the freezing process and there was an associated increase in acrosomal defects and decrease in tail defects.

Figure 30 shows that while the percentage of glycerol in the diluent affected the percentage of live and motile spermatozoa during the freezing process, 4% being least deleterious, it had much less effect on spermatozoal morphology. Increasing level of glycerol, however, did cause a small increase in the percentage of spermatozoa with detached acrosomes.

DISCUSSION

The result of this experiment shows that spermatozoal activity as assessed by live and motility percentages dropped after dilution of the raw semen irrespective of glycerol levels.

Further loss in spermatozoal activity occurred during the stage of equilibration of the diluted semen at $4^{o}C$.

The drop in the spermatozoal activity at these two stages was associated with an increase in acrosomal defects. A similar association between live percentage and acrosomal defects was observed in the preliminary study.

The greatest fall in the spermatozoal activity occurred after freezing followed by thawing, especially after one month's storage in liquid nitrogen and might be due to the great disruption of the spermatozoal metabolism after such processing as observed in ram, bull, boar and rabbit spermatozoa by White et al. (1954); Quinn et al. (1969); O'shea (1969b); Murdoch and O'shea (1973); Darin et al. (1973) and

Darin and Bennett et al. (1973), which in turn leads to the death of large numbers of the frozen spermatozoa. In addition frozen ram as well as human spermatozoa showed some metabolic activity during their storage in liquid nitrogen as observed by Graves (1968) and Ackerman (1968 & 1970), which led to low post thawing spermatozoal motility.

At the same time, release of phospholipid and decrease in DNA and other cytochemical changes in the spermatozoa during the different stages of the deep freezing process, especially after freezing followed by thawing as observed by Mukhergee (1964); Graham and Pace (1967); Platove (1968); Quinn et al. (1969); Nauk et al. (1970); Nauk and Skvorcova (1970); Nath (1972); Darin et al. (1973); Polge and Butler (1973); Coulter and Foote (1975) and Hibbitt and Moore (1975), increased the possibility of the spermatozoal death, associated with loss of motility (Lasley, 1944 and Nour Eldin et al., 1969) and increase in morphological (acrosomal) defects (Bishop et al., 1954; Bishop and Hancock, 1955; Beatty, 1957; Hulet et al., 1965 and Saake and Marshall, 1968). The low post thawing spermatozoal motility irrespective of the glycerol levels, might be due to increased susceptibility of ram spermatozoa to cold shock during storage at $4^{o}C$ as well as freezing when the semen has been collected by means of the electro-ejaculator as observed by Colleary and Ehlers (1964); Fraser (1968); Quinn et al. (1968a); Visser (1969); Moule (1970) and Entwistle and Martin (1972).

Dilution of the ram semen with different levels of glycerol 2-8%, showed that spermatozoal activity (live and motility percentages) throughout the deep freezing process fell as the glycerol level decreased or increased beyond 4% (figure 30). This means that the optimum glycerol level for freezing ram semen was 3.20% of the final dilution which is in

agreement with the finding of Lightfoot and Salamon (1969a), quite near to 3.18% as found by Fraser (1968 & 1970) and fairly near to 4% as found by Colas (1975). This level of glycerol is also in agreement with the findings of Curtis et al. (1961) and Nagase et al. (1964 a & b), in freezing bull semen as well as of Visser and Salamon (1974), in freezing boar semen.

Unsatisfactory post thawing spermatozoal activity (especially motility) after freezing ram semen diluted with low glycerol level (2%) suggests insufficient protection against the freezing damage.

However unsatisfactory post thawing spermatozoal activity (live and motility percentages) after freezing with high glycerol level (8%) might be due to the toxicity of the glycerol at such level. Feredean and Bragaru (1963); Fraser (1968 & 1970) and Colas (1975) observed that glycerol was toxic to ram spermatozoa if the level exceeded 4%. However it is not in agreement with those who claimed that levels of glycerol above 4% (5-15%) were the optima in freezing ram semen (Emmens and Blackshaw, 1950; Hill et al., 1959; First et al., 196la; Aamdal and Andersen, 1968b; Loginova and Zeltsbrjuh, 1968a; Sainsbury, 1968; Visser, 1969, and Vinha and Coubrough, 1972a).

However the optimum glycerol level might vary according to the other components of the diluent (Salamon, 1968) as it varies with different species (Polge et al., 1949). Thus bull semen could be frozen satisfactorily without glycerol (Nagase et al., 1964a & 1968; Salamon, 1968; Pace and Graham, 1974, and Graham, Larson and Crabo, 1974), or with as little as 1.75% (Nagase and Niwa, 1964).

The post thawing live percentages after freezing with low level of glycerol (2%) was greater than those with high glycerol levels (6 and 8%) in spite of the post thawing mobility with 2% glycerol being lower than those with 6% glycerol but slightly higher than those with 8% glycerol. Such differences might be due to the toxicity of 8% glycerol as well as the interference of glycerol with eosin stain being greater with 6 and 8% glycerol than 2%. This observation is in agreement with those who found that theeosinophilic count of diluted semen increased as the glycerol level in the diluent increased (see page 97).

The variation in the post thawing spermatozoal activity with different trials might indicate that glycerol requirement in freezing ram semen differed between rams as well as between ejaculates of the same ram as found by White et al. (1954); Hill et al. (1959); Salamon (1968); and Sainsbury (1968).

Acrosomal damage, detachment and their combination increased slightly as the glycerol level increased from 2 to 8%, during the whole freezing process. This result is similar to that of Smith and Polge (1950a); Almquist (1959); Saacke and Almquist (1962); Marshall et al. (1968); Polge and Butler (1972); Harris et al. (1973); Jones (1973a); Wilmut and Polge (1974) and Yasuda and Tanimura (1974), who found that glycerol decreased acrosomal damage but did not prevent it and the damage increased as the glycerol level increased. On the other hand Jones (1965a & 1972c) claimed that glycerol as well as egg yolk increased the acrosomal defects during cooling and storage of the ram, bull and boar spermatozoa. Besides the high acrosomal damage at 8% glycerol, the live and motility percentage also dropped excessively, an

observation similar to those of Miller and Van Dermark (1953); Blackshaw (1958a); Martin (1963d); Jones (1965b), and Salamon (1968).

In addition to the acrosomal defect, the post nuclear cap became damaged during the different stages of the deep freezing process. This damage has been observed with the electron microscope (figure 69) but its extent could be assessed accurately under the light microscope. Bending as well as looping of the spermatozoal tails was common in the raw semen which might indicate that the rams had a degree of testicular disorder (Fraser and Penman, 1971). The defects decreased somewhat after dilution and equilibration which might be correlated to loss or decrease of spermatozoal activity (motility and live percentages). After freezing and storage the tail defects dropped considerably when there was a related sharp drop in spermatozoal activity. But according to Drevius (1963 & 1972) and Drevius and Eriksson(1966),tail defects increase on dilution and storage especially with hypertonic glycerol diluents (Drevis, 1963 and Fraser, 1968).

The decrease in tail defects might be due to the considerable distortion of the midpiece fibrils and the mitochondrial cristae during freezing (figures 59-70) as found by Nath (1972) in freezing ram semen and Koehler (1966) and Leverage et al. (1972) in freezing bull semen which lead to their immobilization.

CONCLUSION

The effect of stages of the freezing process on the spermatozoal activity and their morphology followed the same rules as in the preliminary freezing.

The live and the motility percentages of the spermatozoa were maintained nearly equal during the dilution and equilibration stages when the diluents contain either 2, 4 or 6% glycerol, while after freezing and thawing after 24 hours or one month, the spermatozoa live and motility percentages were highest when the diluent contained 4% glycerol. Throughout the whole freezing process spermatozoa live and motility percent were lowest when the diluent contained 8% glycerol.

The spermatozoa live and motility percentages decreased after each stage. The greatest decrease was during the freezing and storage stages especially after one month.

Spermatozoa acrosomal damage and acrosomal detachment increased at the end of each stage especially on freezing and thawing, and their increase was greater as the glycerol percent increased from 2 to 8% at all stages of the freezing process.

Spermatozoa tail defect percentages fell after each stage especially on freezing and thawing after one month.

As a consequence of these observations, 4% glycerol will be taken as the optimum level for the subsequent experiments where glycerol is the selected cryoprotective.

5) EXPERIMENT NO. 2

THE EFFECT OF EQUILIBRATION TIME IN GLYCEROL DILUENT ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

The optimum equilibration time differs for different sires as well as between ejaculates of the same individual according to Choong and Wales (1963) and Brown and Harris (1963). Bull semen diluted with egg yolk and glycerol required a long equilibration time ranging from 15 to 20 hours as found by Polge and Rowson (1952 a & b); Polge (1953); Blackshaw (1955b); Cragle et al. (1955); Saroff and Mixner (1955) and Nagase and Niwa (1964). On the other hand bull semen could be frozen without equilibration (Blackshaw and Emmens, 1953 and Emmens and Martin, 1957) when the diluent contained a sugar like arabinose.

In comparisons of equilibration times in the freezing of bull spermatozoa there were no differences between 18 hours and 5 minutes (Sherman, 1957) or 18 hours and 1 hour (First et al., 1959b) or 18 hours and 4 hours (Martin, 1963b). The required time of equilibration in freezing ram semen also varied from a very short time such as 15 minutes (Patt and Nath, 1969) up to 18 hours (Vinha and Coubrough, 1972a).

The aim of this experiment was to study the effect of various times of equilibration at 4°C on the viability and the morphology of the ram spermatozoa throughout the freezing process and to determine the optimum equilibration time.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples, were diluted with 25% egg yolk, 71% lactose (11% solution), and 4% glycerol, at a rate of 1:4 (semen:diluent) to obtain 3.20% glycerol at final dilution. Dilution was carried out directly at 37°C in a water bath.

Following the necessary evaluation, the diluted semen was kept in the refrigerator at 4°C for 0.5, 1.5, 3.0, 4.5, 6.0, 12.0 and 24.0 hours of equilibration.

At the end of each time, part of the equilibrated semen was frozen on dry ice -79°C followed by storage in liquid nitrogen (-196°C).

Thawing of the frozen pellets was done in a dry test-tube in hot water at 60°C after 24 hours and one month.

Evaluation of motility was carried out immediately after dilution, at the end of each time of equilibration, and following thawing after ultra low temperature storage (-196°C) for 24 hours and one month.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defect. Six replicates were evaluated for each time of equilibration.

RESULTS

The counts of the different variables studied in the raw and processed semen samples, are shown as percentages in table A16 in the appendix.

The analyses of variance of the data for the processed semen are presented in table A17 to A22, in the appendix.

The mean values of the different parameters in the six raw and diluted semen samples are presented in table 23. The mean percentages of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% level are shown in table 24 - live, 25 - motility, 26 - acrosomal damage, 27 - acrosomal detachment, 28 - acrosomal defects, and 29 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 31 and as affected by equilibration time in figure 32.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell slightly, but not significantly from 84.58 ± 3.811 to 82.83 ± 3.909.

On equilibration of the diluted samples, the mean percentage of live spermatozoa fell again from 82.83 ± 3.909 to 70.89 ± 1.454. There was a significant difference ($P < 0.05$) between diluted and equilibrated semen irrespective of equilibration time.

Analysis of variance of the percentage of live spermatozoa (table A17) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to equilibration time.

3) There was a significant interaction ($P < 0.001$) between equilibration time and stage.

1) Freezing of the equilibrated samples, followed by thawing after 24 hours caused a marked and significant ($P < 0.05$) fall in live percentage.

Further marked reduction in the live percentage was found on thawing after one month's storage, when it differed significantly ($P < 0.05$) from those of both previous stages.

2) The highest live percentage was found in samples equilibrated for 3.0 hours followed by 4.5, 1.5, 12.0, 6.0, 24.0 and 0.5 hours. Live percentage in samples equilibrated for 3 hours differed significantly ($P < 0.05$) from that in samples equilibrated for 0.5 hour.

3) Following equilibration, live percentage decreased with equilibration time. Live percentages in samples equilibrated for 0.5 hour differed significantly ($P < 0.05$) from those in samples equilibrated for 24.0 hours.

On thawing after 24 hours storage, the highest live percentage was found in samples equilibrated for 12.0 hours followed by 3.0, 4.5, 1.5, 24.0, 6.0 and 0.5 hours. At this stage the live percentage in samples equilibrated for 1.5, 3.0, 4.5, 12.0 and 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 hour.

On thawing after one month's storage, the highest live percentage was found in samples equilibrated for 3.0 followed by 4.5, 6.0, 1.5, 24.0, 12.0 and 0.5 hours. At this stage the live percentage in samples equilibrated for 3.0, 4.5 and 6.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 hour.

The fall in live percentage from equilibration to thawing, was greatest in samples equilibrated for 0.5 hour, followed by 6.0, 1.5, 4.5, 3.0, 24.0 and 12.0 hours when thawed after 24 hours, and followed by 1.5, 12.0, 6.0, 4.5, 3.0 and 24.0 hours when thawed after one month.

The drop in live percentage from equilibration to thawing after 24 hours and one month's storage was significant ($P < 0.05$) for all equilibration times, except in the case of 12.0 hours where the difference was significant only after one month's storage. There were no significant differences between samples stored for 24 hours and one month, except in the case of samples equilibrated for 1.5 or 12.0 hours.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly but not significantly from 71.67 ± 7.491 to 70.00 ± 6.325.

On equilibration of the diluted semen samples, the mean percentage of motile spermatozoa fell markedly from 70.00 ± 6.325 to 42.26 ± 2.790. There was a significant difference ($P < 0.05$) between the motility percentage of the diluted semen and semen equilibrated for 3 hours or longer.

Analysis of variance of the percentage of motile spermatozoa (table A18) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.01$) attributable to equilibration time.

3) There was a significant interaction ($P < 0.01$) between equilibration time and stage.

1) Freezing of the equilibrated samples, followed by thawing after 24 hours and one month's storage, caused further significant ($P < 0.05$) reductions in the percentage of motile spermatozoa, but there was no significant difference due to storage time.

2) The highest motility percentage was found in samples equilibrated for 1.5 followed by 3.0, 0.5, 6.0, 4.5, 12.0 and 24.0 hours. Motility percentage in samples equilibrated for 0.5, 1.5 and 3.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 24.0 hours.

3) Following equilibration, motility percentage decreased with equilibration time. Motility percentages in samples equilibrated for 0.5 hour differed significantly ($P < 0.05$) from those equilibrated for 4.5 to 24.0 hours. Motility percentages in samples equilibrated for 1.5 hours differed significantly ($P < 0.05$) from those equilibrated for 12.0 or 24.0 hours.

On thawing after 24 hours storage, the highest motility percentage was found in samples equilibrated for 1.5 followed by 3.0, either 0.5 or 6.0, 4.5, 12.0 and 24.0 hours. At this stage, the motility percentage in samples equilibrated for either 1.5 or 3.0 hours differed significantly ($P < 0.05$) from those equilibrated for 24.0 hours.

On thawing after one month's storage, the highest motility percentage was found in samples equilibrated for 3.0 hours followed by 1.5, 6.0, 4.5, 12.0, 0.5 and 24.0 hours. At this stage the motility percentage in samples equilibrated for 3.0 hours differed significantly ($P < 0.05$) from those equilibrated for 24.0 hours.

The drop in motility percentage from equilibration to thawing was greatest in samples equilibrated for 0.5 hour followed by 4.5, 6.0, 1.5, 24.0, 12.0 and 3.0 hours when thawed after 24 hours and followed by 1.5, either 4.5 or 24.0, either 3.0 or 6.0 and 12.0 hours when thawed after one month. The drop in spermatozoal motility percentages from equilibration to thawing after 24 hours and one month's storage was significant ($P < 0.05$) in samples equilibrated for 0.5, 4.5 and 6.0 hours.

The drop in spermatozoal motility percentage from equilibration to thawing after one month's storage was significant ($P < 0.05$) in samples equilibrated for 1.5, 3.0, 12.0 and 24.0 hours.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal damage fell very slightly, but not significantly from 3.75 ± 1.216 to 3.67 ± 0.997.

On equilibration of the diluted semen samples, the mean percentage of acrosomal damage increased markedly from 3.67 ± 0.997 to 9.99 ± 0.590. There were significant differences ($P < 0.05$) between the counts for diluted semen and semen equilibrated for all times from 0.5 to 24 hours.

Analysis of variance of the percentages of spermatozoa with damaged acrosomes (table A19) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There was no significant difference attributable to equilibration time.

3) There was no significant interaction between equilibration time and stage.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage, caused further increases in the acrosomal damage percentages which differed significantly ($P < 0.05$) from that of the equilibrated samples.

However, variation attributable to equilibration time both overall and following equilibration, thawing after 24 hours and thawing after one month was small.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal detachment increased markedly from 3.08 ± 1.028 to 6.67 ± 1.487. The difference between the two means was significant ($P < 0.05$).

On equilibration of the diluted semen samples, the mean percentage of acrosomal detachment increased markedly from 6.67 ± 1.487 to 13.81 ± 1.009. There were significant differences ($P < 0.05$) between the counts for diluted semen and semen equilibrated for 0.5, 1.5, 3.0, 12.0 and 24.0 hours.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A20) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to equilibration time.

3) There was no significant interaction between equilibration time and stage.

1) Freezing of the equilibrated samples, followed by thawing after 24 hours storage caused a further increase in the acrosomal detachment percentage, when it differed significantly ($P < 0.05$) from that of the equilibration stage. The greatest increase in the acrosomal detachment percentage was found on thawing after one month's storage, where it differed significantly ($P < 0.05$) from those of both previous stages.

2) Acrosomal detachment percentage increased with equilibration time.

Acrosomal detachment percentage in samples equilibrated for 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 to 6.0 hours.

Acrosomal detachment percentages in samples equilibrated for 4.5 to 12.0 hours differed significantly ($P < 0.05$) from that in samples equilibrated for 0.5 hours as did that in samples equilibrated for 12.0 hours from that in samples equilibrated for 1.5 hours.

3) After equilibration as well as thawing after one month's storage, acrosomal detachment percentages increased with equilibration time. In the case of thawing after 24hours storage they increased as the equilibration time increased from 0.5 to 3.0 hours followed by 6.0, 4.5, 12.0 and 24.0 hours.

Following equilibration, acrosomal detachment percentage in samples equilibrated for 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 to 4.5 hours. There were also significant differences between samples equilibrated for 6.0 or 12.0 and 0.5 hours and for 12.0 and 1.5 hours.

On thawing after 24 hours storage, acrosomal detachment percentage in samples equilibrated for 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 to 6.0 hours and in samples equilibrated for 12.0 hours from those equilibrated for 0.5 or 1.5 hours.

On thawing after one month's storage, acrosomal detachment percentage in samples equilibrated for 24.0 hours differed significantly ($P < 0.05$) from those equilibrated for 0.5 to 4.5 hours. In samples equilibrated for 12.0 hours it differed significantly from those in samples equilibrated for 0.5 to 3.0 hours and in samples equilibrated for 3.0 to 6.0 hours they differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 and 1.5 hours.

The increase in acrosomal detachment percentage from equilibration to thawing after 24 hours storage was greatest in samples equilibrated

for 4.5 hours followed by 3.0, 24.0, 0.5, 1.5, 12.0 and 6.0 hours but none of these increases was significant.

The increase in acrosomal detachment percentage from equilibration to thawing after one month's storage was greatest in samples equilibrated for 12.0 hours followed by 4.5, 1.5, 24.0, 6.0, 3.0 and 0.5 hours. The difference was significant ($P < 0.05$) in all cases. There was significant differences ($P < 0.05$) between acrosomal detachment percentages in the one month and 24 hour thawings in the case of samples equilibrated for 1.5, 4.5, 6.0, 12.0 and 24.0 hours.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased markedly from 6.83 ± 2.216 to 10.33 ± 1.824. The differences between the two means was significant ($P < 0.05$).

On equilibration of the diluted semen samples, the mean percentage of acrosomal defects also increased markedly from 10.33 ± 1.824 to 23.80 ± 1.035. There were significant differences ($P < 0.05$) between the count for diluted semen and semen equilibrated for all times from 0.5 to 24.0 hours.

Analysis of variance of the percentages of acrosomal defects (table A21) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to equilibration time.

3) There was no significant interaction between equilibration time and stage.

1) Freezing of the equilibrated samples, followed by thawing after 24 hours storage, caused a further increase in the acrosomal defect percentage, when it differed significantly ($P < 0.05$) from that of the equilibration stage. The greatest increase in acrosomal defects percentage was found on thawing after one month's storage, when it differed significantly ($P < 0.05$) from those of both previous stages.

2) Acrosomal defects percentages increased with equilibration time. Percentages in samples equilibrated for 12.0 and 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 to 3.0 hours.

There were also significant differences between samples equilibrated for 4.5 or 6.0 and 0.5 hours, and for 6.0 and 1.5 hours.

3) Following the stage of equilibration, acrosomal defect percentages increased with equilibration time. At this stage acrosomal defect percentages in samples equilibrated for 12.0 or 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 to 3.0 hours and those in samples equilibrated for 4.5 or 6.0 hours differed significantly ($P < 0.05$) from that in samples equilibrated for 0.5 hour.

On thawing after 24 hours storage acrosomal defects percentage increased as the equilibration time increased from 0.5 to 4.5 hours followed by 12.0, 6.0 and 24.0 hours. At this stage, acrosomal defects in samples equilibrated for 3.0 to 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 0.5 hours and defects in samples equilibrated for 6.0, 12.0 and 24.0 hours differed significantly ($P < 0.05$) from those in samples equilibrated for 1.5 hours.

On thawing after one month's storage the highest percentage of defects was found in samples equilibrated for 12.0 hours followed by 24.0, 6.0, 4.5, 1.5, 3.0 and 0.5 hours. At this stage percentages in samples equilibrated for 4.5 to 24.0 hours differed significantly ($P < 0.05$) from that in samples equilibrated for 0.5 hour and percentages in samples equilibrated for 12.0 and 24.0 hours, differed significantly ($P < 0.05$) from that in samples equilibrated for 3.0 hours.

The increase in acrosomal defect percentage from equilibration to thawing after 24 hours storage was greatest in samples equilibrated for 3.0 hours followed by 6.0, 0.5, 1.5, 4.5, 24.0 and 12.0 hours, The differences between these two stages was significant ($P < 0.05$) in samples equilibrated for 0.5, 1.5, 3.0 and 6.0 hours.

The increase in acrosomal defect percentage from equilibration to thawing after one month's storage was greatest in samples equilibrated for 1.5 hours followed by 12.0, 0.5, 4.5, either 3.0 or 6.0, and 24.0 hours and these increases were significant ($P < 0.05$) for all equilibration times. However the acrosomal defect percentage at thawing after one month's storage differed significantly ($P < 0.05$) from that after 24 hours storage only in samples equilibrated for 1.5 hours.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoal tail defects increased slightly from 10.42 ± 3.285 to 13.58 ± 3.244 but the difference was not significant.

On equilibration of the diluted semen samples, the mean percentages of spermatozoal tail defects decreased from 13.58 ± 3.244 to 6.49 ± 0.485, but the difference was not significant.

Analysis of variance of the percentages of spermatozoa with tail defects (table A22) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There was no significant difference attributable to equilibration time.

3) There was no significant interaction between equilibration time and stage.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage, caused a further continuous reduction in the tail defects percentages, when they differed significantly ($P < 0.05$) from that of the equilibration stage. However, variations attributable to equilibration time both overall and following equilibration, thawing after 24 hours and thawing after one month, were small.

Figure 31 shows that there was a decrease in the percentage of live and motile spermatozoa throughout the freezing process. The decrease in motility percentage was greater than that of live percentage especially after equilibration. There was an associated increase in acrosomal defects and decrease in tail defects.

Figure 32 shows that the different equilibration times affected the percentage of live and motile spermatozoa, during the freezing process, 3.0 and 1.5 hours respectively being least deleterous. Morphological spermatozoal defects increased with equilibration time.

DISCUSSION

The result of this experiment shows that the post thawing live and motility percentages were not effectively maintained by short

equilibration of 0.5 hour. This might be due to the low permeability of the cell membrane to glycerol (Ashwood-Smith, 1961) affording reduced protection of the spermatozoa against freezing and thawing damage. In addition 0.5 equilibration is not enough time for acclimatization of ram spermatozoa to the new environment of 4°C according to Birillo and Puhaljskii (1936); Chang and Walton (1946); Mann and Lutwak-Mann(1955) and Walton (1957). Poor post thawing spermatozoal viability following 0.5 hour equilibration was also observed by Jones (1965b) and Jones and Martin (1965). Such a result is not however in agreement with the finding that adequate equilibration time for freezing ram semen is 15.0 minutes (Patt and Nath, 1969) or 0.5 hour (Blackshaw et al., 1957 and Hill et al., 1959).

Equilibration for more than 3 hours on the other hand resulted in lowered post thawing motility which might be due to general weakness of the spermatozoa with ageing as well as cytochemical changes during their storage at 4°C for such periods (see the preliminary study). Unlike motility percentages, the post thawing live percentages varied little with equilibration from 1.5 hours up to 24.0 hours. The reduction of the spermatozoal activity was associated with progressive increase in the acrosomal defects as the equilibration time increased from 0.5 to 24.0 hours, and this is nearly similar to the observations of First et al. (1959b); Hill et al. (1959) and Jones (1972 a & c and 1973a). Therefore overnight equilibration of ram semen as recommended by Szumowski et al. (1956); Visser (1969) and Vinha and Coubrough (1972a) is not advisable.

The optimal equilibration times according to this experiment were 1.5 or 3.0 hours as judged by the post thawing motility percentages and 3.0 or 4.5 hours as judged by the post thawing live percentages, and 3.0 hours was superior in both cases, which is in agreement with the finding of Kuprijanova (1962) and Entwistle and Martin (1972), and near to the finding of Martin (1968); Lightfoot and Salamon (1969a) and Salamon (1970) who claimed that equilibration over 4 hours was not desirable.

CONCLUSION

From the overall findings it can be concluded that equilibration of the diluted semen, outwith a narrow range of 1.5 - 4.5 hours, did not provide satisfactory results after freezing, as judged by post-thawing viability of the spermatozoa. Acrosomal defects, moreover, increased with equilibration time and remained higher in the longer equilibrated samples throughout subsequent freezing and storage.

The optimal equilibration time was concluded to be 3.0 hours.

6) EXPERIMENT NO. 3

THE EFFECT OF DILUTION RATE IN GLYCEROL DILUENT ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

The rate of semen dilution has to be related to the spermatozoal density (Holt, 1953a), spermatozoal activity and the diluent composition (Salamon, 1968). However excessive dilution (over 20 fold) of ram semen is harmful to the spermatozoa (Blackshaw, 1953 and Martin, 1968) and causes a marked decrease in their metabolic activities (Mann and Lutwak Mann, 1948). Dilution rates of 1:4 to 1:8 were the most suitable rates for freezing ram semen according to Platov (1965); Fraser (1968); Aamdal and Andersen (1968a); Lightfoot and Salamon (1969a); Jones (1969b) and Andersen and Aamdal (1972).

The aim of this experiment was to study the effect of various dilution rate, throughout the freezing process, on the spermatozoal viability and morphology of ram semen and to determine the optimum dilution rate.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples, were split into six equal volumes in 10ml. tubes. The diluent of 4% glycerol, 71% lactose (11% solution) and 25% egg yolk was added directly, at 37°C water bath in ratios of 1:1, 1:2, 1:4, 1:6, 1:8 and 1:10 (semen:diluent). After completing the necessary evaluations the tubes containing the diluted semen were kept in the refrigerator at 4°C for three hours equilibration, then frozed and stored as before.

Thawing of the frozen pellets was done in a dry test tube in hot water at 60°C after 24 hours and one month.

Evaluation of motility was carried out immediately after dilution, equilibration, and following thawing after ultra low temperature storage (-196°C) for 24 hours and one month. Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defects. Six replicates were evaluated for each rate of dilution.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A23 in the appendix.

The analyses of variance of the data for processed semen are presented in tables A24-A29 in the appendix.

The mean values of the different parameters of the six raw semen samples are presented in table 30.

The mean percentages of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% level are shown in table 31 - live, 32 - motility, 33 - acrosomal damage, 34 - acrosomal detachment, 35 - acrosomal defects, and 36 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 33 and as affected by the dilution rate in figure 34.

Live Spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa, fell from 75.08 $\pm$ 3.702 to 62.78 $\pm$ 2.367. There were significant differences ($P < 0.05$) between the counts for raw semen and semen diluted from 1:2 to 1:10.

Analysis of variance of the percentage of live spermatozoa (table A24) showed the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to dilution rate.

3) There was no significant interaction between dilution rate and stage.

1) At the end of equilibration of the diluted semen samples, there was a further reduction in live percentage, and a significant difference ($P < 0.05$) between the two stages.

Freezing of the equilibrated samples followed by thawing after 24 hours storage caused a further drop in spermatozoa live percentage, but at this stage it differed significantly ($P < 0.05$) from that of the dilution stage only.

A more marked reduction in live percentage was found on thawing after one month's storage, when it differed significantly ($P < 0.05$) from those of both the dilution and equilibration stages.

2) The highest live percentage was found in samples diluted 1:2 followed by 1:4, 1:1, 1:8, 1:6 and 1:10.

Following dilution, the highest live percentage was found in samples diluted 1:1 followed by 1:2, 1:6, 1:4, 1:8 and 1:10.

Following the equilibration the highest live percentage was found in samples diluted 1:4 followed by 1:2, 1:1, 1:6, 1:10 and 1:8.

On thawing the highest live percentage was found in samples diluted at 1:2 followed by 1:4, 1:6, 1:1, 1:10 and 1:8; when thawed after 24 hours storage, and by 1:4, 1:8, 1:1, 1:6 and 1:10 when thawed after one month's storage.

3) Following the stages of dilution, equilibration and thawing after one month's storage, there were no significant differences attributable to dilution rate. Following thawing after 24 hours storage however live percentage in samples diluted 1:2 differed significantly ($P < 0.05$) from that in samples diluted at 1:6.

The highest drop in spermatozoa live percentage from dilution to equilibration was found in samples diluted 1:1 followed by 1:2, 1:6, 1:8, 1:10 and 1:4. The difference was significant ($P < 0.05$) at all rates of dilution except 1:4.

The highest drop in live percentage from the equilibration to thawing after 24 hours storage was found in samples diluted 1:6 followed by 1:4, 1:1, 1:10, 1:8 and 1:2. The difference was significant ($P < 0.05$) in samples diluted 1:6 only. The highest drop in live percentage from equilibration to thawing after one month's storage was found in samples diluted 1:10 followed by 1:4, 1:6, 1:1, 1:2 and 1:8. The differences were significant ($P < 0.05$) in samples diluted 1:4 and 1:10.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell from 71.67 ± 6.009 to 57.22 ± 2.929. There were significant differences ($P < 0.05$) between the motility percentages in

raw and diluted semen at all rates of dilution.

Analysis of variance of the percentage of motile spermatozoa (table A25) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to dilution rate.

3) There was a significant interaction ($P < 0.001$) between dilution rate and stage.

1) At the end of equilibration of the diluted semen samples, there was a marked reduction in motility percentage, and a significant difference ($P < 0.05$) between the two stages.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused further progressive reduction in motility percentage. The motility percentages after thawing differed significantly ($P < 0.05$) from those of the diluted and equilibrated stages.

2) The highest spermatozoa motility percentage was found in samples diluted 1:4 followed by 1:6, 1:8, 1:2, 1:10 and 1:1. There was a significant difference ($P < 0.05$) between samples diluted 1:4 and 1:1.

3) Following dilution, the highest motility percentage was found in samples diluted 1:2 followed by 1:1, 1:4, either 1:6 or 1:8 and 1:10, but there were no significant differences between means.

Following equilibration, the highest motility percentage was found in samples diluted 1:4 followed by 1:6, 1:2, 1:8, 1:10 and 1:1. Motility percentage in samples diluted 1:4 differed significantly ($P < 0.05$) from that in samples diluted 1:1.

On thawing the highest motility percentage was found in samples diluted 1:4 followed by 1:6, 1:8, 1:10, 1:2 and 1:1, when thawed after 24 hours storage; and by 1:6 or 1:10, 1:8, 1:2, and 1:1, when thawed after one month's storage. There were significant differences ($P < 0.05$) between samples diluted 1:4 and 1:1 or 1:2; and between samples diluted 1:6 and 1:1 when thawed after 24 hours storage.

The highest drop in motility percentage from dilution to equilibration was found in samples diluted 1:1 followed by 1:2, 1:8, 1:10, 1:4 and 1:6. The difference between these stages was significant ($P < 0.05$) at all dilution rates except 1:6.

The highest drop in motility percentages from equilibration to thawing after 24 hours storage was found in samples diluted 1:2 followed by 1:1, 1:6, 1:4, 1:10 and 1:8. The differences between these stages were significant ($P < 0.05$) only at dilution rates 1:1 and 1:2. The highest drop in motility percentage from equilibration to thawing after one month's storage was found in samples diluted 1:6 followed by 1:4, 1:2, 1:1, 1:8 and 1:10. The differences were significant ($P < 0.05$) at all dilution rates except 1:10.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoal acrosomal damages increased from 9.08 ± 2.434 to 12.32 ± 1.154. There was a significant difference ($P < 0.05$) between counts for raw semen and semen diluted at all rates from 1:2 to 1:10.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A26) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There were no significant differences attributable to dilution rate.

3) There was no significant interaction between dilution rates and stage.

1) At the end of equilibration of the diluted semen samples, there was a further increase in the acrosomal damage percentage, but there was no significant difference between the two stages.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage also caused a further increase in acrosomal damages percentage, which in both cases differed significantly ($P < 0.05$) from that following dilution.

Variation in acrosomal damage attributable to dilution rate was small both overall and following the several stages of the freezing process.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with detached acrosomes increased slightly from 2.42 ± 0.664 to 3.81 ± 1.546. There were significant differences ($P < 0.05$) between counts in raw semen and semen diluted 1:6, 1:8 and 1:10.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A27) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

1) At the end of equilibration of the diluted semen samples, there was a further increase in the acrosomal detachment percentages, but there was no significant difference between the two stages.

Freezing of the equilibrated samples followed by thawing also caused a further increase in acrosomal detachment percentage. There was a significant difference ($P < 0.05$) between the stage of dilution and thawing after 24 hours and between the stages of both dilution and equilibration and thawing after one month.

2) The highest acrosomal detachment percentage was found in samples diluted 1:8 followed by 1:10, 1:6, 1:4, 1:2 and 1:1. There were significant differences ($P < 0.05$) between samples diluted 1:6 or 1:10 and 1:1. There were also significant differences ($P < 0.05$) between samples diluted 1:8 or 1:10 and 1:2.

3) Following dilution, the highest acrosomal detachment percentage was found in samples diluted 1:8 followed by 1:10, 1:6, 1:4, 1:2 and 1:1.

Following equilibration, the highest acrosomal detachment percentage was found in samples diluted 1:8 followed by 1:6, 1:4, 1:10, 1:2 and 1:1.

However acrosomal detachment percentages did not differ significantly between dilution rates after either of these stages.

On thawing after 24 hours storage, the highest acrosomal detachment percentage was found in samples diluted 1:8 followed by 1:6, 1:10, 1:4, 1:1 and 1:2. There were significant differences ($P < 0.05$) between samples diluted 1:8 and 1:1 or 1:2 and between samples diluted 1:6 and 1:2.

On thawing after one month's storage, acrosomal detachments increased as the dilution rate increased from 1:1 to 1:10. There were significant differences ($P < 0.05$) between samples diluted 1:8 or 1:10 and 1:1 or 1:2 and between samples diluted 1:10 and 1:4.

The greatest increase in acrosomal detachment percentage from dilution to equilibration was found in samples diluted 1:4 followed by 1:6, 1:8, 1:10, 1:2 and 1:1 but there were no significant differences.

The greatest increase in acrosomal detachment from equilibration to thawing after 24 hours storage was found in samples diluted 1:8 followed by 1:1 or 1:6, 1:10, 1:4 and 1:2, but the differences between these stages were not significant.

The greatest increase in acrosomal detachment percentages from equilibration to thawing after one month's storage was found in samples diluted 1:10 followed by 1:8, 1:6, 1:1, 1:2 and 1:4. The differences between these two stages were significant ($P < 0.05$) in the case of 1:1, 1:6, 1:8 and 1:10 dilution rates.

The increases from dilution to thawing were significant in all cases.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased from 11.50 ± 2.729 to 16.12 ± 1.067. There were significant differences ($P < 0.05$) between counts for raw semen and semen diluted 1:2 to 1:10.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A28) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

1) At the end of equilibration of the diluted semen samples, there was a marked increase in acrosomal defect percentage, the difference being significant ($P < 0.05$).

Freezing of the equilibrated samples followed by thawing after 24 hours and one month, caused a further marked increase in acrosomal defects, the differences being significant ($P < 0.05$) in both cases.

2) Acrosomal defect percentage increased as dilution rate increased from 1:1 to 1:10. There were significant differences ($P < 0.05$) between samples diluted 1:6 to 1:10 and either 1:1 or 1:2.

3) Following dilution, the highest percentage of acrosomal defect was found in samples diluted 1:10 followed by 1:6, 1:8, 1:4, 1:2 and 1:1, but there were no significant differences between means.

Following the stage of equilibration, the highest total acrosomal defects was found in samples diluted 1:10 followed by 1:8, 1:6, 1:4, 1:1 and 1:2. There was a significant difference ($P < 0.05$) between samples diluted 1:10 and 1:2 only.

On thawing after 24 hours storage, the highest percentage of acrosomal defects was found in samples diluted 1:6 followed by 1:8, 1:4, 1:10, 1:2 and 1:1, but there were no significant differences between means.

On thawing after one month's storage, the highest total acrosomal defects was found in samples diluted 1:10 followed by 1:8, 1:6, 1:2, 1:4 and 1:1. There was a significant difference ($P < 0.05$) between samples diluted 1:10 and 1:1 to 1:4.

The greatest increase in the acrosomal defect percentage from dilution to equilibration was found in samples diluted 1:8 followed by 1:10, 1:6, 1:4, 1:1 and 1:2. The differences between these two stages were significant in all dilutions except 1:2.

The greatest increase in the acrosomal defect percentage from equilibration to thawing after 24 hours was found in samples diluted 1:2 followed by 1:4, 1:6, 1:8, 1:1 and 1:10. The difference between these two stages was significant ($P < 0.05$) in dilutions 1:2, 1:4, 1:6 and 1:8.

The greatest increase in total acrosomal defect percentages from equilibration to thawing after one month's storage was found in samples diluted 1:10 followed by 1:2, 1:8, 1:6, 1:1 and 1:4. The difference between these two stages was significant in all dilutions. However, the difference between storage times was significant ($P < 0.05$) only in samples diluted 1:10.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoa with tail defects increased slightly from 4.33 ± 1.188 to 6.25 ± 0.710, but the difference was not significant.

Analysis of variance of the percentage of spermatozoa with tail defects (table A29) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to dilution rate.

3) There was no significant interaction between the dilution rates and stage.

1) At the end of equilibration of the diluted semen samples, there was a slight increase in the tail defect percentage, but freezing of the equilibrated semen samples followed by thawing after 24 hours and one month's storage caused a slight reduction.

2) The highest percentage of tail defects was found in samples diluted 1:6 followed by 1:10, 1:8, 1:4, 1:2 and 1:1. There was a significant difference ($P < 0.05$) between samples diluted 1:6 and 1:1 only.

3) Following dilution, the highest tail defect percentage was found in samples diluted 1:6 followed by 1:10, 1:2, 1:8, 1:4 and 1:1, but the difference between means was not significant.

Following equilibration, the highest tail defect percentage was found in samples diluted 1:10 followed by 1:6, 1:2, 1:4 and either 1:1 or 1:8. There were significant differences ($P < 0.05$) between samples diluted 1:10 and either 1:1 or 1:8.

On thawing after 24 hours storage, the highest tail defects percentage was found in samples diluted 1:8 followed by 1:6, 1:10, 1:4, 1:2 and 1:1, but there was no significant difference between means.

On thawing after one month's storage, the highest tail defect percentage was found in samples diluted 1:6 followed by 1:8, 1:10, 1:4, and 1:2. There were significant differences ($P < 0.05$) between samples diluted 1:6 and either 1:1 or 1:2.

Figure 33 shows that there was a decrease in the percentage of live and motile spermatozoa throughout the freezing process. The decrease in motility percentage was greater than in live percentage and the difference between them increased following each stage of the freezing process. There was an associated increase in acrosomal defects but tail defects showed little change.

Figure 34 shows that dilution rate did not affect live percentages to any extent although the lower rates gave best results but did affect motility percentage during the freezing process, 1:4 and 1:6 dilution being least deleterous.

Spermatozoal morphology defects increased slightly with dilution rate.

DISCUSSION

The results of this experiment indicate that the different dilution rates 1:1 to 1:10 did not affect the post thawing live percentages very much but dilution rates of 1:2 and 1:4 gave the highest scores and 1:6 dilution rate was significantly inferior to 1:2 ($P < 0.05$). Such differences might be exaggerated due to the higher rate of acrosomal damage at the higher dilution rates, which in turn facilitates the passage of eosin stain (Dott and Walton, 1960).

On the other hand the post thawing motility was adversely affected by dilution rates as low as 1:1 and 1:2 and to some extent by dilution rates as high as 1:8 and 1:10. Dilution rate of 1:1 was inferior to 1:4 and 1:6 ($P < 0.05$), and 1:2 was also inferior to 1:4 ($P < 0.05$). Differences between rates were evident by the end of equilibration.

Dilution rates of 1:6 and especially 1:4 were found to be best for freezing ram semen which is in agreement with the findings of Platov (1965); Lightfoot and Salamon (1969a); Jones (1971a); Salamon and Visser (1972); Visser (1974a) and Mielikovic et al. (1974).

The unsatisfactory post thawing motility in samples diluted at a low rate (1:1 - 1:2) might be due to lactic acid accumulation, especially when lactose is included in the diluent, which leads to reduction in the pH. (Amir, Schindler, Eyal, Lehrer and Kempenich-Pinto, 1973). Visser (1969) has also observed reduction in motility with alteration in pH.

Dilution rates of 1:6, 1:8 and 1:10 increased both acrosomal and tail defects in association with the reduced live and motility percentages. This might indicate that the spermatozoa of the ram becomes more susceptible to freezing damages and cold shock, like those of the boar, when diluted at rates of 1:6 or 1:10 (Pursel, Johnson and Schulman, 1973a) or over 1:8 (Salamon, 1973 and Bower, Crabo, Pale and Graham, 1973), as well as of the bull when diluted over 1:8 (Saacke, Robbins and Chandler, 1974). High dilution rate of ram spermatozoa at 1:10 or over was harmful during cooling or deep freezing (Blackshaw, 1953; Fraser, 1968; Martin, 1968 & 1972 and Andersen, Aamdal and Fougner, 1973) either by increasing the permeability of the cell membrane (Dott and Walton, 1960) or causing mechanical injuries. On the other hand motility percentages were adversely affected by the lowest dilution rate of 1:1, possibly due to insufficient cryoprotection by the resulting 2% level of glycerol.

CONCLUSION

The highest post thawing live percentages were obtained with a dilution rate of 1:2 or 1:4 and the highest motility percentages with 1:4 and 1:6. In addition acrosomal defects increased as the dilution rate increased from 1:1 to 1:10. Therefore, a dilution rate of 1:4 is considered to be best and will be used in the following experiment.

7) EXPERIMENT NO. 4

THE EFFECT OF THAWING MEDIUM ON FROZEN RAM SPERMATOZOA STORED AT -196°C

INTRODUCTION

Redilution of thawed ram semen in saline solutions (First, et al., 1961a) or sugars (Jones and Martin, 1965; Lightfoot and Salamon, 1969 a & b and Salamon and Brandon, 1971), and of bull semen in yolk citrate (Hafs and Elliott, 1954) improved the post thawing motility of the spermatozoa. However Desjardins and Hafs(1962) suggest that it is advisable to re-extend thawed bull semen in a medium different from that used for freezing of the raw samples.

The aim of this experiment was to study the effect of various thawing media on the viability and morphology of frozen ram spermatozoa and determine the optimum thawing medium, if any.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples were diluted 1:4 with diluent composed of 25% egg yolk, 71% lactose (11% solution) and 4% glycerol, (3.2% glycerol in final dilution). Dilution was carried out directly at 37°C in a water bath. Following the necessary evaluations the diluted semen was kept in the refrigerator at 4°C for 3 hours followed by freezing and storage in liquid nitrogen (-196°C). Thawing of the frozen pellet was done in hot water at 60°C after 24 hours and one month with the following media:-

1) Sodium chloride solution 0.9% at 37°C.

2) Frozen pellets of sodium chloride solution 0.9% at -196°C.

3) Sodium citrate solution 3% at 37°C.

4) Frozen pellet of Sodium citrate solution 3% at -196°C.

5) Equal parts of sodium citrate solution 3% + lactose solution 11% at 37°C.

6) Frozen pellets of equal parts of sodium citrate solution 3% + lactose solution 11% at -196°C.

7) Dry test tube (control).

The dilution during thawing was 2:3 (frozen semen: thawing medium). Evaluation of motility was carried out immediately after dilution, at the end of equilibration time, and following thawing after ultra low temperature storage (-196°C) for 24 hours and one month.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentages of live spermatozoa and morphological defects. Six replicates were evaluated for each time of thawing.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A30 in the appendix.

The analyses of variance of the data for processed semen are presented in tables A31-A36 in the appendix.

The mean values of the different parameters in the six raw, diluted and equilibrated samples are presented in table 37.

The mean percentage of the different variables in the processed semen samples on thawing after storage for 24 hours and one month, the standard errors of their differences and the significant differences at the 5% level are shown in table 38 - live, 39 - motility, 40 - acrosomal damage, 41 - acrosomal detachment, 42 - acrosomal defects and 43 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 35 and as affected by thawing medium in figure 36.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell slightly, but not significantly from 71.25 $\pm$ 4.381 to 69.00 $\pm$ 3.152.

On equilibration of the diluted semen samples, the mean percentage of live spermatozoa fell again from 69.00 $\pm$ 3.152 to 64.83 $\pm$ 3.759 this difference being significant ($P<0.05$).

On freezing of the equilibrated semen samples followed by thawing with different thawing media after 24 hours and one month's storage the mean percentage of live spermatozoa fell from 64.83 $\pm$ 3.759 to 50.64 $\pm$ 2.213 and 33.36 $\pm$ 2.213 respectively. In both cases, the differences were significant ($P<0.05$) irrespective of thawing medium.

Analysis of variance of the percentage of live spermatozoa (table A31) shows the following:-

1) There was a significant difference ($P<0.01$) attributable to storage time of the frozen semen.

2) There was a significant difference ($P<0.05$) attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

1) The highest live percentage following 24 hours and one month's storage combined was found in samples thawed with medium No. 7 (no medium) followed by 4, 5, 6, 3, 1 and 2.

2) On thawing after 24 hour's storage the highest live percentage was found in samples thawed with medium No. 7 followed by 5, 4, 6, 1, 3 and 2.

On thawing after one month's storage the highest live percentage was found in samples thawed with medium No. 4 followed by 7, 5, 6, 2, 3, and 1.

However none of the differences between the means for thawing media was significant with the statistical tests used here.

The greatest difference in live percentage between storage times was found in samples thawed with medium No. 1 followed by 5, 6, 3, 7, 4 and 2.

The difference was significant ($P \leq 0.05$) in the cases of samples thawed with medium Nos. 1, 3, 5 and 6.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly but not significantly from 65.00 ± 5.00 to 58.33 ± 4.014.

On equilibration of the diluted semen samples, the mean percentage of motile spermatozoa fell again from 58.33 ± 4.014 to 50.83 ± 6.380. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing with different medium after 24 hours and one month's storage the mean percentage of motile spermatozoa fell from 50.83 ± 6.380 to 28.69 ± 2.413 and 10.71 ± 2.413 respectively. There were significant differences ($P < 0.05$) between the motility percentages of the equilibrated semen and frozen semen thawed with medium Nos. 1, 2, 3, 5, 6 and 7 after 24 hour's storage and with all thawing media after one month's storage.

Analysis of variance of the percentage of motile spermatozoa (table A32) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to storage time of the frozen semen.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and the storage time.

1) The highest motility percentage was found in samples thawed with medium No. 4 followed by 5, 3, 7, 6, 1 and 2.

2) On thawing after 24 hour's storage the highest motility percentage was found in samples thawed with medium No. 5 followed by 4, 3, 7, 6, 2 and 1. The difference between samples thawed in media Nos. 5 and 1 was significant ($P < 0.05$).

On thawing after one month's storage, the highest motility percentage was found in samples thawed with medium No. 4 followed by 7, 6 either 1 or 3, 5 and 2, but none of the differences was significant.

The greatest difference in motility percentage between storage times was found in samples thawed with medium No. 5 followed by 3, 2, 4 and either 1, 6 or 7. The difference was significant ($P < 0.05$) in samples thawed with media Nos. 2, 3, 4 and 5.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with damaged acrosomes increased slightly but not significantly from 3.42 ± 0.625 to 5.25 ± 0.981.

On equilibration of the diluted semen samples, the mean percentage increased further from 5.25 $\pm$ 0.981 to 10.33 $\pm$ 1.711. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing with different media after 24 hours and one month's storage, the mean percentage increased further from 10.33 $\pm$ 1.711 to 13.42 $\pm$ 0.526 and 13.73 $\pm$ 0.526 respectively. There was a significant difference ($P < 0.05$) between counts for the equilibrated semen and frozen semen thawed with media Nos. 5 and 7 after 24 hours storage.

Analysis of variances of the percentage of spermatozoa with damaged acrosomes (table A33) shows the following:-

1) There was no significant difference attributable to storage time of the frozen semen.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with detached acrosomes increased slightly but not significantly from 1.42 $\pm$ 0.327 to 2.17 $\pm$ 0.628.

On equilibration of the diluted semen samples, the mean percentage increased again from 2.17 $\pm$ 0.628 to 5.17 $\pm$ 0.997. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing with different media after 24 hours and one month's storage, the mean

percentage increased further from 5.17 $\pm$ 0.997 to 10.90 $\pm$ 0.485 and 14.71 $\pm$ 0.485 respectively. In both cases, the difference was significant ($P < 0.05$) irrespective of thawing medium.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A34) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to storage time of the frozen semen.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

The greatest difference in acrosomal detachment percentages between storage times was found in samples thawed with medium No. 2 followed by 4, 3, 1, either 5 or 7, and 6. The differences were significant ($P < 0.05$) in the cases of media Nos. 1, 2, 3 and 4.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of acrosomal defects increased significantly ($P < 0.05$) from 4.83 $\pm$ 0.703 to 7.42 $\pm$ 1.248.

On equilibration of the diluted semen samples, the mean percentage increased again from 7.42 $\pm$ 1.248 to 15.50 $\pm$ 1.643. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing with different thawing media after 24 hours and one month's storage, the mean percentage of acrosomal defect increased further from 15.50 $\pm$ 1.643 to 24.32 $\pm$ 0.582 and 28.44 $\pm$ 0.582 respectively. In both cases

the differences were significant ($P < 0.05$) irrespective of thawing medium.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A35), shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to storage time of the frozen semen.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

The greatest difference in acrosomal defect percentage between storage times was found in samples thawed with medium No. 1 followed by 3, 4, 7, 5 and 2 or 6. The differences were significant ($P < 0.05$) in the cases of media Nos. 1 and 3.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoal tail defects showed no change.

On equilibration of the diluted semen samples, the mean percentages of tail defects increased slightly, but not significantly from 5.08 ± 1.977 to 8.75 ± 2.960.

On freezing of the equilibrated semen samples followed by thawing with different thawing media after 24 hours and one month's storage, the mean percentage tail defects decreased from 8.75 ± 2.960 to 6.44 ± 1.084 and 3.92 ± 1.089 respectively. There was a significant difference ($P < 0.05$) between counts for the equilibrated semen and semen thawed with medium No. 4 after 24 hours storage.

Analysis of variance of the percentage of spermatozoa with tail defects (table A36) shows the following:-

1) There was no significant difference attributable to storage time of the frozen semen.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Figure 35 shows that there was a decrease in percentage of live and motile spermatozoa throughout the freezing process. Motility percentage decreased more than live percentage and the difference between them increased following each stage of the freezing process. There was an associated increase in acrosomal defects, but tail defects showed little change.

Figure 36 shows that live percentage was least adversely affected by thawing media Nos. 4 and 7, and motility percentage by media Nos. 3 and 7. Morphology was practically unaffected.

DISCUSSION

The result of this experiment indicates that the post thawing motility was improved somewhat by redilution with media containing Sodium citrate as found by Visser and Salamon (1974); Platov (1965); Salamon (1968) and Lightfoot and Salamon (1969 a & b) for ram semen and Martin (1965b) for bull semen. Inclusion of sugar (lactose) gave similar results and this is in agreement with Jones and Martin (1965); Lightfoot and Salamon (1969a & b); Salamon and Brandon (1971) and Salamon (1973). Such improvement might be due to the correction of any

alterations in the pH which arise during storage before freezing (Mann, 1964). Lactose, by renourishment of the spermatozoa, was claimed to enhance motility by Salamon and Lightfoot (1969) and Salamon (1970), but the effect was inconsistent here.

The post thawing motility and live percentages were lowest after redilution with sodium chloride.

The post thawing motility and live percentages were higher when thawing media containing sodium chloride or sodium citrate plus lactose were added to the frozen semen as a solution at 37°C rather than as frozen pellets at -196°C. Such a result is in agreement with the observations of O'shea (1969a) who found that thawed ram and bull spermatozoa survived well after redilution and incubation at 37°C. Similarly Pursel and Johnson (1975) observed that the best way of thawing boar semen was to add the thawing solution prewarmed up to 50° C to the frozen semen.

In case of sodium citrate alone, however, the post thawing live and motility percentages were higher when added to the frozen semen as a frozen pellet than as a solution at 37°C, especially after one month's storage.

However the overall result indicates that there was no significant difference between the different thawing media and this is in agreement with the observations of Page et al. (1968a); Nagase et al. (1964a) and Salamon (1968 & 1970) who found no significant difference between various thawing media such as egg yolk, milk, citrate or chloride on the post thawing survival in bull and ram semen. Dry thawing, however, gave the highest post thawing live percentage. In addition it avoids the possibility of any harmful effect of excessive redilution, especially

when the prefreezing dilution rate is above 3 folds which made dry thawing preferable according to Salamon (1973); Salamon et al. (1973) and Visser (1974a).

Spermatozoal morphological defects were scarcely affected at all by the different thawing media, but acrosomal defects were slightly higher in the case of dry thawing and this is in agreement with Salamon (1973); Salamon et al. (1973) and Visser (1974 a & b).

CONCLUSION:

The various media, either at 37°C or in the form of frozen pellets, added to frozen pellets of ram semen at the time of thawing, had little effect on the post-thawing characteristis of the spermatozoa when compared with the simple control method of thawing pellets in a dry test tube at 37°C.

8) EXPERIMENT NO. 5

THE EFFECT OF THAWING TEMPERATURE ON THE FROZEN RAM SPERMATOZOA STORED AT -196°C

INTRODUCTION

The optimum temperature for thawing of frozen semen differs according to species and individuals, (Dunn et al., 1953). From the literature so far studied, it has been found that some prefer a slow thawing rate at low temperature, from 0°C in ice water (Hill et al., 1959 and Boyd and Hafs, 1968) to 5°C (Dunn et al., 1953 and Miller and Van Demark, 1953 & 1954), while others prefer a fast thawing rate at body temperature (Salamon, 1967, 1968 & 1971 and Entwistle and Martin, 1972), or higher up to water boiling point (Aamdal and Andersen, 1968 a & b).

The aim of this experiment was to study the effect of various thawing temperatures from 0°C to 100°C, on the viability and morphology of frozen ram spermatozoa, and to determine the optimum thawing temperature.

MATERIALS AND METHODS

Part of the frozen semen of the previous experiment (No. 4) was used for the purpose of this experiment.

Thawing of the frozen pellets in a dry test tube was done at 0°C, 20°C, 37°C, 45°C, 60°C, 80°C and 100°C, after 24 hours storage in liquid nitrogen (-196°C).

Evaluation of motility was carried out immediately after each thawing.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defects. Six replicates were evaluated for each thawing temperature.

RESULTS

The counts of the different variables studied in the processed semen samples are shown as percentages in table A37 in the appendix.

The analyses of variance of the data for processed semen are presented in table A38 in the appendix.

The mean percentage of the different variables in the processed semen samples at the different thawing temperatures, the standard error of their differences and the significant differences at the 5% level are shown in table 44.

The relationship of the different variables as affected by thawing temperature is illustrated in figure 37.

Live spermatozoa

On freezing of the equilibrated semen samples followed by thawing after 24 hours storage at different thawing temperatures, the mean percentage of live spermatozoa fell from 64.83 ± 3.759 to 48.87 ± 3.484; the difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of live spermatozoa (table A38) shows that there was a significant difference ($P < 0.05$) attributable to thawing temperature.

The highest live percentage was found in samples thawed at 60°C followed by 100°C, 37°C, 45°C, 80°C, 20 and 0°C. However, despite the

result of analysis of variance no pair of means was found to differ significantly with the statistical test used here.

Motile spermatozoa

The mean percentage of motile spermatozoa fell from 50.83 $\pm$ 6.379 to 20.00 $\pm$ 3.728, the difference between means being significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of motile spermatozoa (table A38) shows that there was a significant difference ($P < 0.05$) attributable to thawing temperature.

The highest motility percentage was found in samples thawed at 37°C followed by 60°C, 45°C, 80°C, 100°C, 20° and 0°C. The difference was significant ($P < 0.05$) only between samples thawed at 0°C and 37°C.

Spermatozoa with damaged acrosomes

The mean percentage of spermatozoa with acrosomal damage increased from 10.33 $\pm$ 1.711 to 17.50 $\pm$ 2.012. There were significant differences ($P < 0.05$) between the means for equilibrated semen and for semen thawed at 20°C, 45°C, 60°C, 80°C and 100°C.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A38) shows that there was no significant difference attributable to thawing temperature but the highest percentage was found in samples thawed at 80°C followed by 100°C, 45°C, 20°C, 0°C, 37°C and 60°C.

Spermatozoa with detached acrosomes

The mean percentage of spermatozoal acrosomal detachment increased from 5.17 $\pm$ 0.997 to 10.43 $\pm$ 0.997, the difference being significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A38), shows that there was no significant difference attributable to thawing temperature but the highest percentage was found in samples thawed at 80°C followed by 100°C, 60°C, 45°C, 20°C, 0°C and 37°C.

Total spermatozoa with acrosomal defects

The mean percentage of spermatozoa with acrosomal defects increased from 15.50 ± 1.643 to 27.93 ± 1.716. There were significant differences between the means for equilibrated semen and for semen thawed at all temperatures except 0°C.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A38), shows that there was no significant difference attributable to thawing temperature but the highest percentage found in samples thawed at 80°C, followed by 100°C, 45°C, 20°C, 0, 60°C and 37°C.

Spermatozoa with tail defects

The mean percentages of spermatozoal tail defects decreased slightly but not significantly from 8.75 ± 2.960 to 7.02 ± 1.015.

Analysis of variance of the percentage of spermatozoa with tail defects (table A38) shows that there was no significant difference attributable to thawing temperature but the highest tail defect was found in samples thawed at 37°C followed by 60°C, 0°C, 80°C, 20, 100°C and 45°C.

Figure 37 shows that live and motility percentages were affected to some degree by the different thawing temperatures, the least deleterous being in the higher range from 37 to 100°C.

Spermatozoal morphology was adversely affected to some extent by thawing temperatures of 80 and 100°C.

DISCUSSION

The result of this experiment indicates that post thawing spermatozoal live percentages were better when the frozen semen was thawed at 37 to 100°C (fast thawing) than at 0 and 20°C (slow thawing). Similarly post thawing spermatozoal motility percentages were best with thawing at 37 to 60°C but thawing at 0 and 20°C was still inferior to 80 and 100°C. Such observations agree with others that frozen ram semen has to be thawed fast e.g. at 37°C (Jones and Martin, 1965; Sainsbury, 1968; Lightfoot and Salamon, 1969a; Salamon, 1971; Vinha and Coubrough, 1972a and Entwistle and Martin, 1972), or 45°C (Salamon, 1970), or higher up to boiling water (Aamdal and Andersen, 1968 a & b; Andersen and Aamdal, 1972; Almquist and Wiggen, 1973 a & b; Saack et al., 1974; Wiggin and Almquist, 1975 a & b; Olar, Becker and Senger, 1975 and Senger, Becker, Gerber and Hillers, 1975). Slow thawing, especially at 0°C (Hill et al., 1969) was not preferred. During thawing at 20°C spermatozoa will pass through the zero temperature and thereafter rise to 20°C. Iype, Abraham and Bhargava (1963) observed that warming of bull and buffalo semen from 0°C to room temperature (20°C) caused marked acrosomal damage and protein loss. The unfavourable result after thawing at 0°C and 20°C would be in agreement with their findings but are in disagreement with those who prefer thawing temperatures of 5°C (Miller and Van Demark, 1954) or 20°C (Zakrzewska, 1962).

Spermatozoal morphological defects were not affected much by the different thawing temperature but to some extent acrosomal defects

increased with increased thawing temperature over 60°C, (Saacke and White, 1972; Robbins et al.,1972; Saacke et al.,1974 and Wiggin and Almquist, 1975a).

CONCLUSION

The post thawing spermatozoal live, motility and morphological defect percentages did not differ significantly throughout the different thawing temperatures but fast thawing at or above 37°C was better than slow thawing. As thawing at 60°C gave the highest percentage of live sperms, and did not appear to be associated with the increase in acrosomal defects seen at yet higher temperatures, it is preferred.

9) EXPERIMENT NO. 6

THE EFFECT OF DIMETHYL SULPHOXIDE ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

Among other cryoprotectives which have been used in freezing living tissue, is dimethyl sulphoxide (DMSO), (Lovelock and Bishop, 1959; Ashwood-Smith, 1961; Porterfield and Ashwood-Smith, 1962; Dougherty, 1962; Sherman, 1964; Jones, 1965b and Richardson and Sadleir, 1967).

DMSO has a protective action equal to that of glycerol but it is more toxic to the spermatozoa during storage at 5°C or freezing according to Sherman (1964); Jones (1965b) and Richardson and Sadleir (1967).

The optimal level of DMSO ranged between 2.5 to 10% in freezing human spermatozoa (Sherman, 1964 and Richardson and Sadleir, 1967), and 3 to 9%, 3% being best, in freezing ram semen (Jones, 1965b).

The aim of this experiment is to study the effect of level of DMSO in the diluent on ram spermatozoa during deep freezing and to determine the optimum percentage.

MATERIALS AND METHODS

These were exactly as in experiment No. 1, except for the substitution of glycerol by dimethyl sulphoxide (DMSO).

RESULTS

The counts of the different variables studied in the raw and processed semen samples, are shown as percentages in table A39 in the appendix.

The analyses of variance of the data for the processed semen are presented in tables A40–A45 in the appendix.

The mean values of the different parameters in the six raw semen samples are presented in table 45.

The mean percentages of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% level are shown in tables 46 - live, 47 - motility, 48 - acrosomal damages, 49 - acrosomal detachment, 50 - acrosomal defects and 51 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 38 and as affected by level of dimethyl sulphoxide (DMSO) in figure 39.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell slightly from 94.75 ± 2.016 to 93.50 ± 3.153. There were significant differences ($P < 0.05$) between the counts for raw semen and semen diluted with 6 or 8% DMSO.

Analysis of variance of the percentage of live spermatozoa (table A40) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to DMSO level.

3) There was no significant interaction between DMSO level and stage.

1) At the end of equilibration of the diluted semen samples there was a further, but not significant reduction in live percentage.

Freezing of the equilibrated samples followed by thawing after 24 hours storage caused a marked drop in live percentage which differed significantly ($P < 0.05$) from those of the dilution and equilibration stages.

Freezing of the equilibrated samples followed by thawing after one month's storage caused the greatest reduction in the live percentage which differed significantly ($P < 0.05$) from those of the previous stages, and from that on thawing after 24 hours storage.

2) Live percentage decreased as the DMSO level increased from 2 to 8%, but there were no significant differences between means.

3) Following dilution, the highest percentage was found in samples diluted with 4% DMSO followed by 2, 8 and 6%, while following the stage of equilibration, live percentage decreased as the DMSO level increased from 2 to 8%. However at these two stages, there were no significant differences between means.

On thawing after 24 hours storage, the highest live percentage was found in samples diluted with 4% DMSO followed by 2, 6 and 8%. There was a significant difference ($P < 0.05$) only between samples diluted with 4 and 8% DMSO.

On thawing after one month's storage, live percentage decreased as the DMSO level increased from 2 to 8%. There were significant differences between samples diluted with 2 and 6 or 8% DMSO.

The greatest but not significant drop in live percentage from dilution to equilibration was found in samples diluted with 8% DMSO followed by 4, 6 and 2%.

The greatest drop in live percentage from equilibration to thawing after 24 hours storage was found in samples diluted with 8% DMSO,

followed by 6, 2 and 4%. The differences were significant ($P < 0.05$) in the cases of 6 and 8%.

The greatest drop in live percentage from equilibration to thawing after one month's storage was found in samples diluted with 6% DMSO, followed by 8, 4 and 2%. The differences were significant ($P < 0.05$) in all cases.

In addition there was a significant difference ($P < 0.05$) between samples after 24 hours and one month's storage irrespective of DMSO level.

Motile spermatozoa

On dilution of the raw semen the mean percentage of motile spermatozoa fell slightly but not significantly from 78.33 ± 3.073 to 76.67 ± 3.168.

Analysis of variance of the motility percentages (table A41) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.01$) attributable to DMSO level.

3) There was a significant interaction ($P < 0.05$) between the DMSO level and stage.

1) At the end of equilibration of the diluted samples there was a further significant ($P < 0.05$) reduction in motility percentage.

Freezing of the equilibrated semen samples followed by thawing after 24 hour's storage caused a great reduction in motility percentages. The motility percentage at this stage differed significantly ($P < 0.05$) from those of both the dilution and equilibration stages.

Freezing of the equilibrated semen samples followed by thawing after one month's storage caused the greatest reduction in the motility percentage, which differed significantly ($P < 0.05$) from those of the previous stages, and that on thawing after 24 hour's storage.

2) The greatest drop in motility percentage throughout the freezing process was found in samples diluted with 8% DMSO followed by 6, 2 and 4%. There were significant differences between samples diluted with 2 or 4 and 8% DMSO.

3) Following dilution, the drop in motility percentage was the same irrespective of DMSO level, while following equilibration, motility percentage decreased as DMSO level increased from 2 to 8% but there were no significant differences between means.

On thawing after 24 hour's storage, the highest motility percentage was found in samples diluted with 4% DMSO followed by 6, 2 and 8%. There were significant differences ($P < 0.05$) between samples diluted with 2, 4 or 6 and 8% DMSO.

On thawing after one month's storage, the highest motility percentage was found in samples diluted with 4% DMSO followed by 2, 6 and 8%. There was a significant difference ($P < 0.05$) between samples diluted with 4 and 8% DMSO.

The drop in motility percentage from dilution to equilibration increased as the DMSO level increased from 2 to 8%.

The greatest drop in motility percentage from equilibration to thawing after 24 hour's storage was found in samples diluted with 8% DMSO followed by 2, 6 and 4%.

The greatest drop in motility percentage from equilibration to thawing after one month's storage was found in samples diluted with

6 or 8% DMSO followed by 4 and 2%.

Motility percentages differed significantly ($P < 0.05$) between the stages of dilution , equilibration and thawing irrespective of DMSO level, and there was also a significant difference ($P < 0.05$) between storage times in case of samples diluted with 4 or 6% DMSO.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with damaged acrosomes increased slightly but not significantly from 3.08 ± 0.712 to 3.63 ± 1.349.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A42), shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There was no significant difference attributable to DMSO level.

3) There was no significant interaction between the DMSO level and stage.

1) At the end of equilibration of the diluted semen samples there was a small,but not significant, increase in acrosomal damage percentage.

Freezing of the equilibrated semen samples followed by thawing after 24 hour's storage caused a further increase in spermatozoal acrosomal damage percentage, which differed significantly ($P < 0.05$) only from that following dilution.

Freezing of the equilibrated semen samples followed by thawing after one month's storage caused the greatest increase in acrosomal damage percentages which differed significantly ($P < 0.05$) from those of both previous stages.

2) The highest acrosomal damage percentage was found in samples diluted with 8% DMSO followed by 2, 4 and 6%.

3) Variation attributable to DMSO level following the stages of dilution, equilibration and thawing after 24 hours and one month's storage were very small, the ranges being 3.50-3.92%, 6.67-8,83%, 8.08-10.83% and 11.33-14.67% respectively.

The greatest increase in the acrosomal damage percentage from dilution to equilibration was found in samples diluted with 8% DMSO followed by 4, 6 and 2%.

The greatest increase in the acrosomal damage percentage from equilibration to thawing after 24 hour's storage was found in samples diluted with 2% DMSO followed by 4, 8 and 6%.

The greatest increase in the acrosomal damage percentage from the stage of equilibration to the stage of freezing followed by thawing after one month's storage was found in samples diluted with 2 or 4% DMSO followed by 8 and 6%.

However the only significant differences ($P < 0.05$) were those between percentages after dilution and on thawing after one month's storage irrespective of DMSO level.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with detached acrosomes increased slightly from 2.17 ± 0.900 to 3.63 ± 0.605. There was a significant difference ($P < 0.05$) between counts for raw and diluted semen irrespective of DMSO level.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A43) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.01$) attributable to DMSO level.

3) There was no significant interaction between the DMSO level and stage.

1) At the end of equilibration of the diluted semen samples there was a marked increase in the acrosomal detachment percentages.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused further continuous increases in the acrosomal detachment percentage.

The differences were significant ($P < 0.05$) in all cases.

2) Acrosomal detachment increased as the DMSO levels increased from 2 to 8%, but the difference was significant ($P < 0.05$) only between samples diluted with 2 and 8% DMSO.

3) Following dilution the highest acrosomal detachment percentages were found in samples diluted with 4 or 8% DMSO followed by 2 or 6%. While following equilibration, the highest percentage was found in samples diluted with 6% DMSO followed by 8, 4 and 2%. However, at these two stages, there were no significant differences between means.

On thawing after both 24 hours and one month's storage, acrosomal detachment percentage increased as the DMSO level increased from 2 to 8%. In both cases the difference was significant ($P < 0.05$) only between samples diluted with 2 and 8% DMSO.

The greatest increase in acrosomal detachment percentage from dilution to equilibration was found in samples diluted with 6% DMSO followed by 8, 4 and 2%. The differences were significant ($P < 0.05$)

in all cases.

The greatest increase in acrosomal detachment percentage from equilibration to thawing after both 24 hours and one month's storage was found in samples diluted with 8% DMSO followed by 2, 4 and 6% The increase was significant ($P < 0.05$) in all cases except for samples diluted with 6% DMSO from equilibration to thawing after 24 hours.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of acrosomal defects increased slightly from 5.25 ± 1.430 to 7.25 ± 1.374. There was a significant difference ($P < 0.05$) between counts for raw semen and semen diluted with 4 or 8% DMSO.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A44) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to DMSO level.

3) There was no significant interaction between the DMSO level and stage.

1) At the end of equilibration of the diluted semen samples there was a marked increase in acrosomal defects.

Freezing of the equilibration semen samples followed by thawing after 24 hours and one month's storage caused a further increase in acrosomal defects.

The differences were significant ($P < 0.05$) in all cases.

2) The highest acrosomal defect percentage was found in samples diluted with 8% DMSO followed by 4, 2 and 6%. There were significant differences ($P < 0.05$) between samples diluted with 2 or 6% and 8% DMSO.

3) Following each stage of the freezing process, the highest total acrosomal defect percentage was found in samples diluted with 8% DMSO followed by 4, 6 and 2% after dilution; by 6, 4 and 2% after equilibration; by 2, 4 and 6% after freezing followed by thawing after 24 hour's storage, and by 4, 2 and 6% after freezing followed by thawing after one month's storage. However the differences were significant ($P < 0.05$) only between samples diluted with 6 and8% on thawing after 24 hour's storage and with 2 or 6 and 8% on thawing after one month's storage.

Acrosomal defect percentages from dilution to equilibration increased as the DMSO level increased from 2 to 8%. The difference between stages was significant ($P < 0.05$) in each case.

The greatest increase in acrosomal defect percentage from equilibration to thawing after 24 hour's storage was found in samples diluted with 2% DMSO followed by 8, 4 and 6%. The differences were significant ($P < 0.05$) in the case of 2 and 8% DMSO.

The greatest increase in acrosomal defects percentage from equilibration to thawing after one month's storage was found in samples diluted with 8% DMSO followed by 2, 4 and 6%. The differences were significant ($P < 0.05$) in all cases.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of tail defects increased slightly, but not significantly from 17.58 ± 8.548 to 18.17 ± 2.159.

Analysis of variance of the percentage of spermatozoa with tail defects (table A45) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to DMSO level.

3) There was a significant interaction ($P < 0.05$) between DMSO level and stage.

1) At the end of equilibration of the diluted semen samples there was a marked and significant reduction ($P < 0.05$) in the tail defect percentage.

Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage led to a further but not significant reduction.

2) The highest percentage of tail defects was found in samples diluted with 6% DMSO followed by 2, 8 and 4%. The difference was significant ($P < 0.05$) only between samples diluted with 4 and 6% DMSO.

3) Following dilution, the highest tail defect percentage was found in samples diluted with 4% DMSO followed by 8, 2 and 6%. The differences were significant ($P < 0.05$) between samples diluted with 2, 4 or 8% and 6% DMSO.

Variations due to DMSO level following equilibration and thawing after 24 hour's and one month's storage, were small, the ranges being 6.08-8.75%, 2.33-5.58% and 1.50-3.17% respectively.

The greatest reduction in tail defect percentage from dilution to equilibration was found in samples diluted with 4% DMSO followed by 2, 8 and 6%. The differences were significant ($P < 0.05$) except in the

case of samples diluted with 6% DMSO.

The greatest reduction in tail defect percentages from equilibration to thawing was found in samples diluted with 8% DMSO, followed by 6, 2 and 4% when thawed after 24 hour's storage, and by 2, 6 and 4% when thawed after one month's storage. However, none of these differences were significant.

Figure 38 shows that there was a decrease in the percentage of live and motile spermatozoa throughout the freezing process. Motility percentage decreased more than live percentage and the difference between them increased with stage, especially freezing followed by thawing after 24 hour's and one month's storage. There was an associated increase in acrosomal defects. Tail defects showed a slight increase after dilution followed by a progressive decrease, especially after equilibration.

Figure 39 shows that live and motility percentages were affected by DMSO level. The least deleterious being 2 and 4%. The percentage of acrosomal defects was highest with 8% DMSO.

DISCUSSION

The results of this experiment show that the use of DMSO instead of glycerol (Experiment 1) made no material difference to the variation between samples, nor did it alter the overall effects of stages (cf. figures 29 & 38). Although motility was less affected by DMSO during dilution and equilibration, the advantage was lost during actual freezing and storage.

Spermatozoal live percentage throughout the freezing process decreased as the Dimethyl sulphoxide (DMSO) level increased from 2 to 8%.

On the other hand spermatozoa motility percentage throughout the freezing process decreased as the Dimethyl sulphoxide decreased or increased beyond 4% (figure 39).

Such findings indicate that the optimal requirement of the DMSO in freezing ram semen was 1.60 or 3.20% (final dilution) which confirms Jones (1965b) who found 3% best in freezing ram semen, and it also confirms Sherman (1964) and Richardson and Sadlier (1967) who found 2.5% DMSO best in freezing human spermatozoa.

Spermatozoal morphological defects were little affected by the different levels of DMSO but the highest number of acrosomal defects was seen with 8% DMSO, as with 8% glycerol in Experiment No. 1.

CONCLUSION

It can be concluded that it is possible to freeze ram semen with either 2 or 4% Dimethyl sulphoxide, in order to obtain reasonable post thawing spermatozoa survival and minimize morphological damage to the spermatozoa. Since there was no evidence that either was superior, a compromise of 3% DMSO is considered optimal.

The general effects of freezing on the spermatozoa were broadly similar to those observed when freezing with glycerol in Experiment No. 1, but a more critical comparison of glycerol and DMSO is made in Experiment No. 11.

10) EXPERIMENT NO. 7

THE EFFECT OF EQUILIBRATION TIME IN DIMETHYL SULPHOXIDE DILUENT ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

The aim of this experiment was the same as in experiment No. 2.

MATERIALS AND METHODS

These were the same as in experiment No. 2 except that the diluent consisted of dimethyl sulphoxide (DMSO) 3%, lactose (11% solution) 72%, and egg yolk 25%.

RESULTS

The counts of the different variables studied in the raw and processed semen samples, are shown as percentages in table A46 in the appendix.

The analyses of variance of the data for the processed semen are presented in tables A47-A52.

The mean values of the different parameters in the six raw and diluted samples are presented in table 52.

The mean percentages of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% levels are shown in tables 53 - live, 54 - motility, 55 - acrosomal damages, 56 - acrosomal detachment, 57 - acrosomal defects and 58 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 40, and as affected by equilibration time in figure 41.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa increased very slightly but not significantly from 77.50 ± 4.612 to 77.75 ± 4.434.

On equilibration of the diluted semen samples, the mean percentage of live spermatozoa fell from 77.75 ± 4.434 to 68.27 ± 1.271. The difference was significant ($P < 0.05$) irrespective of equilibration time.

Analysis of variance of the percentage of live spermatozoa (table A47) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to equilibration time.

3) There was a significant interaction ($P < 0.001$) between equilibration time and stage.

1) Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage, caused a further reduction in live percentage. The differences in percentage between equilibration and both storage times, and between the two storage times were all significant ($P < 0.05$).

2) The highest live percentage was found in samples equilibrated for 1.5 hours followed by 3.0, 12.0, 4.5, 6.0, 24.0 and 0.5 hours, the difference between the two extremes just failing to reach significance ($P < 0.05$).

3) Following equilibration, the highest live percentage was found

in samples equilibrated for 0.5 hour followed by 1.5, 3.0, 6.0, 12.0 4.5 and 24.0 hours, but there were no significant differences.

On thawing after 24 hours storage the highest live percentage was found in samples equilibrated for 1.5 hours followed by 12.0, 3.0 4.5, 24.0, 6.0 and 0.5 hours. The differences between samples equilibrated for 1.5, 3.0 or 12.0 hours and 0.5 hour were significant ($P < 0.05$).

On thawing after one month's storage the highest live percentage was found in samples equilibrated for 3 hours followed by 1.5, 12.0, 4.5, 6.0, 24.0 and 0.5 hours. There were significant differences ($P < 0.05$) between samples equilibrated for 1.5, 3.0 or 12.0 and 0.5 or 24.0 hours and between samples equilibrated for 4.5 or 6.0 and 0.5 hours.

The greatest drop in live percentage from equilibration to thawing was found in samples equilibrated for 0.5 hour, followed by 3.0, 6.0, 1.5, 4.5, 24.0 and 12.0 hours when thawed after 24 hours storage, and by 24.0, 1.5, 6.0, 3.0, 4.5 and 12.0 hours when thawed after one month. The differences were significant ($P < 0.05$) in the cases of samples equilibrated for 0.5, 3.0 and 24.0 hours when thawed after 24 hours storage and all cases after one month's storage

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly but not significantly from 79.17 ± 6.379 to 68.33 ± 6.009.

On equilibration of the diluted samples the mean percentage fell further from 68.33 ± 6.009 to 41.79 ± 1.116. The difference was significant ($P < 0.05$) irrespective of equilibration time.

Analysis of variance of the percentage of motile spermatozoa (table A48) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to equilibration time.

3) There was a significant interaction ($P < 0.001$) between equilibration time and stage.

1) Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused further reduction in motility percentage. The differences in percentage between equilibration and both storage times and between the two storage times were all significant ($P < 0.05$).

2) The highest motility percentage was found in samples equilibrated for 1.5 hours followed by 6.0 or 12.0, 3.0, 4.5, 0.5 and 24.0 hours.

3) Following equilibration, motility percentage decreased with equilibration time. There were significant differences ($P < 0.05$) between samples after 0.5 and 6.0 hours; after 1.5 hours and 12.0 hours; and after 3.0 and 24.0 hours.

On thawing, the highest motility percentage was found in samples equilibrated for 12.0 hours followed by 6.0, 24.0, 1.5, 3.0, 4.5 and 0.5 hours when thawed after 24 hours storage, and by 6.0, 1.5, 4.5, 3.0 and 0.5 or 24.0 hours when thawed after one month's storage. The differences were significant ($P < 0.05$) between samples equilibrated for 6.0, 12.0 or 24.0 and 0.5 hours in the case of thawing after 24 hours

storage, and between samples equilibrated for 12.0 hours and 0.5 or 24.0 hours in the case of thawing after one month's storage.

The greatest drop in motility percentage from equilibration to thawing was found in samples equilibrated for 0.5 hour followed by 1.5, 3.0, or 4.5, 6.0, 12.0 and 24.0 hours when thawed after 24 hours storage and by 1.5, 3.0, 4.5, 24.0, 6.0 and 12.0 hours when thawed after one month's storage. The differences were significant ($P < 0.05$) in the cases of samples equilibrated for 0.5 to 12.0 hours when thawed after 24 hours storage and in all cases when thawed after one month's storage.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with damaged acrosomes increased slightly but not significantly from 5.00 ± 1.667 to 6.92 ± 1.129.

On equilibration of the diluted samples the mean percentage of acrosomal damage increased markedly from 6.92 ± 1.129 to 14.85 ± 0.760. There were significant differences ($P < 0.05$) after all equilibration times except 1.5 hours when the mean was 10.25%.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A49) shows the following:

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to equilibration time.

3) There was no significant interaction between the equilibration time and stage.

Variation throughout the freezing process was small but damage tended to increase with time during equilibration. There were significant differences ($P < 0.05$) between samples equilibrated for 0.5 or 1.5 hours and 24.0 hours.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with detached acrosomes increased slightly but significantly ($P < 0.05$) from 1.75 ± 0.642 to 2.92 ± 0.952.

On equilibration of the diluted semen samples the mean percentage of spermatozoal acrosomal detachments increased from 2.92 ± 0.952 to 10.56 ± 0.578. The difference was irrespective of equilibration time.

Analysis of variance of the percentage of detached acrosomes (table A50) shows the following:-

1) There was a significant difference ($P < C.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to equilibration time.

3) There was no significant interaction between the equilibration time and stage.

1) Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused a further increase in percentage of detached acrosomes. The differences in percentage between equilibration and both storage times and between the two storage times were all significant ($P < 0.05$).

2) Acrosomal detachment increased with equilibration time. The differences between samples equilibrated for 0.5 or 1.5 hours and 12.0

or 24.0 hours and between samples equilibrated for 0.5 and 6.0 hours were significant ($P < 0.05$).

3) After each stage, the highest acrosomal detachment percentage was found in samples equilibrated for 24 hours, followed by 12.0 to 0.5 hours at the end of equilibration; by 12.0 to 3.0, 0.5 and 1.5 hours on thawing after 24 hours storage; and by 6.0, 12.0, 4.5, 1.5, 3.0 and 0.5 hours on thawing after one month's storage. There were significant differences ($P < 0.05$) between samples after 0.5 and 6.0 hours; after 1.5 and 12.0 hours, and after 3.0 or 4.5 and 24.0 hours equilibration; between samples equilibrated for 0.5, 1.5, 3.0, 4.5 or 6.0 and 24.0 hours; and for 1.5 and 12.0 hours on thawing after 24 hours storage; and between samples equilibrated for 0.5 and 6.0 hours and 1.5 or 3.0 and 24.0 hours, on thawing after one month's storage.

The greatest increase in acrosomal detachment percentage from equilibration to thawing after 24 hours storage was found in samples equilibrated for 0.5 hours followed by 24.0, 3.0, 4.5, 6.0, 1.5 and 12.0 hours. The differences were significant ($P < 0.05$) in case of samples equilibrated for 0.5, 3.0, 4.5 and 24.0 hours.

The greatest increase in acrosomal detachment percentage from equilibration to thawing after one month's storage was found in samples equilibrated for 1.5 hours followed by 0.5, 6.0, 4.5, 3.0, 24.0 and 12.0 hours. The differences were significant ($P < 0.05$) in all cases.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased significantly ($P < 0.05$) from 6.75 ± 2.205 to 9.83 ± 1.969.

On equilibration of the diluted semen samples, the mean percentage of spermatozoal total acrosomal defect increased from 9.83 ± 1.969 to 25.40 ± 0.503. The difference was significant ($P < 0.05$) irrespective of equilibration time.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A51) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to equilibration time.

3) There was a significant interaction ($P < 0.05$) between the equilibration time and stage.

1) Freezing of the equilibrated samples followed by thawing after 24 hours and one month's storage caused further increases in the percentages of acrosomal defects, the difference being significant ($P < 0.05$) in both cases.

2) Total acrosomal defects, increased with equilibration time. There were significant differences ($P < 0.05$) between samples equilibrated for 0.5 or 1.5 hours and 4.5, 6.0, 12.0 or 24.0 hours, and for 3.0, 4.5 or 6.0 hours and 24.0 hours.

3) After each stage of the freezing process, the highest percentage of acrosomal defects was found in samples equilibrated for 24 hours, followed by 12.0 to 0.5 hours at the end of equilibration; by 12.0, 4.5, 3.0, 6.0, 1.5 and 0.5 hours on thawing after 24 hours storage, and by 6.0, 12.0, 4.5, 3.0, 0.5 and 1.5 hours on thawing after one month's storage. The differences were significant ($P < 0.05$) between samples

equilibrated for 0.5 and 3.0 hours, for 0.5 or 1.5 and 4.5, 6.0, 12.0 or 24.0 hours and for 3.0 or 4.5 and 24.0 hours at the end of equilibration; between samples equilibrated for 0.5 or 1.5 hours and 12.0 or 24.0 hours; and for 6.0 hours and 24.0 hours equilibration on thawing after 24 hours storage, and between samples equilibrated for 0.5, 1.5, 3.0 or 4.5 and 24.0 hours on thawing after one month's storage.

The greatest increase in acrosomal defect percentage from equilibration to thawing was found in samples equilibrated for 0.5 hours followed by 3.0, 1.5, 4.5, 12.0, 24.0 and 6.0 hours when thawed after 24 hours storage, and by 1.5, 3.0, 4.5, 24.0, 6.0 and 12.0 hours when thawed after one month's storage. The differences were significant ($P < 0.05$) in all cases except for samples equilibrated for 6.0 and 24.0 hours in case of thawing after 24 hours storage and for 12.0 hours in case of thawing after one month's storage.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoal tail defects fell slightly but not significantly from 5.75 ± 2.438 to 4.00 ± 1.784.

On equilibration of the diluted samples, the mean percentage of spermatozoal tail defects increased slightly from 4.00 ± 1.784 to 4.54 ± 0.375. The difference between these stages was significant ($P < 0.05$) only in the case of 6.0 hours equilibration when the mean was 6.33%.

Analysis of variance of the percentage of spermatozoa with tail defects (table A52) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to equilibration time.

3) There was no significant interaction between the equilibration time and stage.

Variations throughout this experiment were very slight, the range being only 3.00 - 6.33%.

Figure 40 shows that there was a continuous decrease in the percentage of live and motile spermatozoa, throughout the freezing process, and the decrease in motility percentage was greater than that of live percentage, although the initial raw semen motility percentage was slightly higher than that of live percentage. There was an associated increase in acrosomal defects and slight decrease in tail defects.

Figure 41 shows that the different equilibration time affected the percentage of live and motile spermatozoa during the freezing process; 1.5 hours was least deleterious. Acrosomal defects increased as the equilibration increased from 1.5 to 24.0 hours, but tail defect increased very slightly.

DISCUSSION

The results of this experiment showed that the percentages of live and motile spermatozoa decreased progressively with equilibration time and there was an associated increase in acrosomal defects. After freezing and thawing, however, the percentages of live and motile spermatozoa were markedly lower in samples equilibrated for 0.5 hour, which clearly allowed insufficient time for DMSO to penetrate the spermatozoa in order to protect them against the freezing damage. Equilibration for as long as 24 hours, on the other hand, was followed by much the poorest results following freezing and storage for one month.

These results are similar to those obtained in experiment No. 2 where glycerol was used in the diluent, except that equilibration for 1.5 hours here was slightly better than equilibration for 3.0 hours. Such a difference, though small, might be due to either faster penetration of the cell membrane by DMSO than by glycerol (Lovelock and Bishop, 1959; Sherman, 1960; Jones, 1965a & b, and Musul'Bas and Vishnevski, 1974) or to the protective mechanism of the DMSO being extracellular (Malinin, Fontana and Braungart, 1968).

Moreover, Clegg, Komarek and Pickett (1965) suggest that the optimum time for equilibration with glycerol could be affected by other factors, among them, the processing time between collection and dilution (Polge, 1957, and Jones, 1965b) or glycerol level (Wilmut et al., 1973) which leads to the need for longer equilibration time with glycerol than with DMSO (Blackshaw, 1960b).

CONCLUSION

This experiment confirmed the results of experiment No. 2, where glycerol was the cryoprotective, that equilibration for as little as 0.5 hour is inadequate for the protection of ram spermatozoa during freezing. Post thawing motility percentage, however, did not fall until samples had been equilibrated for 24 hours, whereas with glycerol they fell in samples equilibrated for more than 3 hours.

Although DMSO allowed a longer range of equilibration time than glycerol, no viability of the thawed semen resulted from equilibration for more than 1.5 hours and moreover acrosomal defects increased with time from 1.5 hours. It is therefore concluded that equilibration for 1.5 hours is optimal for freezing ram spermatozoa in diluent containing DMSO.

11) EXPERIMENT NO. 7a

ACROSOMAL DEFECTS OF SPERMATOZOA IN RAW AND DILUTED SEMEN SAMPLES STORED AT 4°C FROM 0 TO 24.0 HOURS

INTRODUCTION

In Experiment No. 2 it was found that the proportion of spermatozoa with acrosomal defects increased with equilibration time from 0 - 24 hours.

Similar observations were reported by White et al. (1954); First et al. (1959b); Hill et al. (1959); Blackshaw (1960b), and Jones (1972c). The increase might be simply due to ageing or a deleterious effect of the treatment. Willett et al. (1940) claimed that storage of ram semen in vitro was harmful to spermatozoa which did not survive for more than a matter of hours.

Accordingly, the aim of this experiment was to study the effect on the spermatozoal acrosomes of simple storage (ageing) of raw semen at 4°C compared with equilibration with the cryoprotective diluent.

MATERIALS AND METHODS

During the equilibration process (0.5 - 24.0 hours), of the previous experiment (No. 7), part of the raw semen was stored in parallel at 4°C and samples were removed for assessment of acrosomal abnormalities at the same intervals and eosin fast green FCF stain was used. In the case of the diluted semen, 0 hour equilibration was just after dilution of the raw semen.

RESULTS

The counts of the acrosomal damage, acrosomal detachment and the total spermatozoa with acrosomal defects in the raw and diluted semen samples are shown as percentages in tables A53 and A46 respectively in the Appendix.

The analyses of variance of the data for the raw and diluted semen samples are presented in tables A54-56 in the appendix. The mean percentages of the different variables in the raw and the diluted semen samples at equivalent storage and equilibration times, the standard errors of their differences and the significant differences at the 5% levels are shown in tables 59 - acrosomal damage, 60 - acrosomal detachment, and 61 - acrosomal defects.

The relationship of the different variables as affected by time is illustrated in figure 42.

Spermatozoa with damaged acrosomes

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A54) shows the following:-

1) There was no significant difference attributable to treatment of semen.

2) There was a significant difference ($P < 0.001$) attributable to storage time.

3) There was no significant interaction between treatment and storage time.

1) Acrosomal damage percentage in diluted semen was slightly higher than in raw semen.

2) The percentage of damaged acrosomes increased with time. There

were significant differences ($P < 0.05$) between means after 0 and 1.5 hours; after 0.5 and 4.5 hours; after 1.5 and 6.0 hours, and after 3.0 and 24.0 hours.

3) In raw semen there were significant differences ($P < 0.05$) between means after 0 and 4.5 hours; and after 0.5 and 24.0 hours. In diluted semen samples, there was also a progressive increase in percentage with time, except for a slight fall at 12.0 hours. There were significant differences ($P < 0.05$) between means after 0 and 3.0 hours; after 0.5 and 4.5 hours; after 1.5 and 6.0 or 24.0 hours, and after 3.0 and 24.0 hours.

The greatest difference in acrosomal damage percentage between the raw and diluted semen was found in samples stored for 4.5 followed by 6.0, 3.0, 24.0, 12.0, 0, 0.5 and 1.5 hours but none of the differences was significant.

Spermatozoa with detached acrosomes

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A55) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to treatment of semen.

2) There was a significant difference ($P < 0.001$) attributable to storage time.

3) There was a significant interaction ($P < 0.001$) between treatment of semen and storage time.

1) Acrosomal detachment percentage in diluted semen was significantly ($P < 0.05$) greater than in raw semen.

2) Acrosomal detachment percentage increased as storage time increased from 0 to 24 hours. There were significant differences ($P < 0.05$) between means after 0 and 0.5 hours; after 0.5 and 3.0 hours; after 1.5 and 4.5 hours; after 3.0 and 6.0 hours, and after 6.0 or 12.0 and 24.0 hours storage at $4^{o}C$.

3) In both the raw and diluted semen samples, acrosomal detachment percentage increased as the storage times at $4^{o}C$ increased from 0 to 24.0 hours. There were significant differences ($P < 0.05$) between means after 0, 0.5 or 1.5 and 12.0 hours and after 3.0 to 12.0 and 24.0 hours in case of the raw semen.

There were significant differences ($P < 0.05$) between means after 0 and 1.5 hours; after 0.5 and 3.0 hours; after 1.5 and 6.0 hours; after 3.0 or 4.5 and 12.0 hours, and after 6.0 and 24.0 hours in case of diluted semen.

The difference in the acrosomal detachment percentage between the raw and diluted semen increased with time and was greatest in samples stored for 12.0 hours followed by 24.0 and 6.0 to 0 hours. The differences were significant ($P < 0.05$) at all times except 0 hour.

Total spermatozoa with acrosomal defects

Analysis of variance of percentage of spermatozoa with acrosomal defects (table A56) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to treatment of semen.

2) There was a significant difference ($P < 0.001$) attributable to storage time.

3) There was a significant interaction ($P < 0.05$) between treatment of semen and the storage time.

1) Acrosomal defect percentages in diluted semen samples was greater than in raw semen samples.

2) Acrosomal defect percentage increased with time. There were significant differences ($P < 0.05$) between means after 0 and 0.5 to 24.0 hours; after 0.5 and 3.0 to 24.0 hours; after 1.5 and 4.5 to 24.0 hours; after 3.0 and 6.0 to 24 hours and after 4.5 to 12.0 and 24.0 hours.

3) In both the raw and diluted semen samples, acrosomal defects increased with time. There were significant differences ($P < 0.05$) between means after 0 and 1.5 hours; after 0.5 and 6.0 hours; after 1.5 and12.0 hours, and after 3.0 and 24.0 hours in the case of raw semen. There were significant differences ($P < 0.05$) between means after 0 and 1.5 hours; after 0.5 and 3.0 hours; after 1.5 and 4.5 hours; after 3.0 and 12.0 hours, and after 4.5 or 6.0 and 24.0 hours in the case of diluted semen.

The greatest difference in acrosomal defect percentage between raw and diluted semen was found in samples stored for 6.0 hours followed by 4.5, 24.0, 12.0, 3.0, 1.5, 0.5 and 0 hours. The differences were significant ($P < 0.05$) in the cases of storage for 3.0 to 24.0 hours.

Figure 42 shows that the percentages of spermatozoa with acrosomal damage, acrosomal detachment and their total were higher in diluted semen than in raw semen throughout storage. Moreover in both cases they increased with time from 0 to 24.0 hours except that in diluted semen the percentage with acrosomal damage was slightly less at 12.0 hours than at 4.5 or 6.0 hours.

DISCUSSION

The result of this experiment indicated that both the raw and especially the diluted semen samples showed a progressive increase in the acrosomal damage and especially their detachments throughout storage at 4°C. The progressive acrosomal changes in diluted semen stored at 4°C are in agreement with the observations of White et al. (1954), First et al. (1959b), Hill et al. (1959), Blackshaw (1960b) and Jones (1972c).

Dilution of the raw semen at 37°C as well as the storage of the diluted semen at 4°C was found to aggravate the acrosomal changes which is in agreement with observations of Dott and Walton (1960), Jones and Martin (1965), Martin (1968) and Jones (1972a).

The acrosomal changes observed, classed as acrosomal damage (swollen, empty, fractured and multi-defective) followed by acrosomal detachments were similar to the changes in processed semen described as the non-specific reaction by Healey (1969), Nath (1972), Watson and Martin (1972), Jones (1972a & 1973a) and Visser (1974a).

These defects in ram semen, however, might be due to post-mortem changes as described in bull semen by Hancock (1952).

CONCLUSION

Acrosomal defects, in both raw and diluted ram semen increase progressively and directly with time of storage at 4°C, but more so in diluted semen therefore it is necessary to dilute semen promptly after collection and to keep the equilibration time as short as possible without jeopardizing reliability.

12) EXPERIMENT NO. 8

THE EFFECT OF DILUTION RATE IN DIMETHYL SULPHOXIDE DILUENT ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORATE AT -196°C

INTRODUCTION

The aim of this experiment was the same as in experiment No. 3.

MATERIALS AND METHODS

These were the same as in experiment No. 3 except as follows:-

1) The diluent consisted of dimethyl sulphoxide (DMSO) 3%, Lactose (11% solution) 72%, and egg yolk, 25%.

2) The equilibration time was 1.5 hours.

RESULTS

The counts of the different variables studied in the raw and processed semen samples, are shown as percentages in table A57 in the appendix.

The analyses of variance of the data for the processed semen are presented in tables A58 - A63 in the appendix.

The mean values of the different parameters in the six raw semen samples are presented in table 62.

The mean percentage of the different variables in the processed semen samples, the standard errors of their differences and the significant differences at the 5% levels are shown in table 63 - live, 64 - motility, 65 - acrosomal damages, 66 - acrosomal detachment, 67 - total acrosomal defects, and 68 - tail defect.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 43, and as affected by dilution rate in figure 44.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 87.58 $\pm$ 2.917 to 83.14 $\pm$ 1.645. There were significant differences ($P < 0.05$) in the cases of samples diluted 1:2, 1:4 and 1:10, in which the means were 82.92, 82.25 and 81.83 respectively.

Analysis of variance of the percentage of live spermatozoa (table A58) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to dilution rate.

3) There was a significant interaction ($P < 0.001$) between the dilution rate and stage.

1) At the end of equilibration of the diluted semen, there was a large reduction in live percentage, and freezing followed by thawing after 24 hours and one month's storage, caused further reductions.

The differences in live percentage between stages but not between storage time were significant ($P < 0.05$).

2) The highest live percentage (overall) was found in samples diluted 1:1 followed by 1:2, 1:4, 1:10, 1:6 and 1:8. The differences between samples diluted 1:1, 1:2 or 1:4 and 1:8 were significant ($P < 0.05$).

3) Following dilution, the highest live percentage was found in samples diluted 1:1, followed by 1:8, 1:2 or 1:6, 1:4 and 1:10 but at this stage there were no significant differences.

Following equilibration, the highest live percentage was found in samples diluted 1:2, followed by 1:1, 1:4, 1:8, 1:6 and 1:10 and the differences between samples diluted 1:1 or 1:2 and 1:6, 1:8 or 1:10, and between samples diluted 1:4 and 1:10, were significant ($P < 0.05$).

On thawing, the highest live percentage was found in samples diluted 1:4 followed by 1:1, 1:10, 1:2, 1:6 and 1:8 when thawed after 24 hours storage, and by 1:6, 1:2, 1:1, 1:10 and 1:8 when thawed after one month's storage. The differences between samples diluted 1:4 and 1:8 on thawing after 24 hours, and between samples diluted 1:4 and 1:6 or 1:8 on thawing after one month, were significant ($P < 0.05$).

The greatest drop in live percentages from dilution to the end of equilibration was found in samples diluted 1:8 followed by 1:10, 1:6 1:1, 1:4 and 1:2.

The greatest drop in live percentages from equilibration to thawing was found in samples diluted 1:2, followed by 1:1, 1:8, 1:6, 1:4 and 1:10 when thawed after 24 hours storage, and by 1:1, 1:8, 1:4, 1:10 and 1:6 when thawed after one month's storage. All differences between stage means were significant ($P < 0.05$) except between equilibration and thawing after 24 hours in the case of samples diluted 1:10. There was however, no significant difference attributable to storage time.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly but not significantly from 83.33 $\pm$ 2.108 to 75.28 $\pm$ 2.272.

Analysis of variance of the percentage of motile spermatozoa (table A59) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

1) At the end of equilibration of the diluted semen, there was a marked reduction in motility percentages and freezing of the equilibrated semen after 24 hours and one month's storage, caused further reductions.

The differences in live percentage between stages but not between storage times were significant ($P < 0.05$).

2) The highest motility percentage was found in samples diluted 1:4 followed by 1:6, 1:2, 1:10, 1:1 and 1:8. The difference between samples diluted 1:4 and 1:8 was significant ($P < 0.05$).

3) Following dilution the highest motility percentage was found in samples diluted at a rate of 1:1 followed by 1:2 or 1:4, 1:6, 1:8 and 1:10.

Following equilibration, the highest motility percentages were found in samples diluted at a rate of 1:4 or 1:6 followed by 1:10, 1:2 or 1:8 and 1:1.

On thawing, the highest motility percentages were found in samples diluted at a rate of 1:4 followed by 1:6, 1:10, 1:2, 1:8 and 1:1 when thawed after 24 hours storage and by 1:6, 1:2 and 1:1, 1:8 or 1:10 when thawed after one month. However, in no case was there a significant difference attributable to dilution rate.

The greatest drop in motility percentage from dilution to the end of equilibration was found in samples diluted 1:1 followed by 1:2, 1:4 or 1:8, 1:6 and 1:10.

The greatest drop in motility percentages from equilibration to thawing after 24 hours storage were found in samples diluted 1:6 followed by 1:1 or 1:10, 1:4 or 1:8 and 1:2.

The greatest drop in motility percentage from equilibration to thawing after one month's storage was found in samples diluted 1:10 followed by 1:6, 1:4 or 1:8, 1:1 and 1:2.

The difference between stage means was significant ($P < 0.05$) in every case but there was no significant difference attributable to storage time.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal damage decreased slightly but not significantly from 2.17 ± 0.997 to 1.69 ± 0.806.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A60) shows the following:-

1) There was a significant difference ($P < 0.05$) attributable to stage of the freezing process.

2) There was no significant difference attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

At the end of equilibration of the diluted semen, there was a slight increase in acrosomal damage percentage, and freezing followed by thawing after 24 hours and one month's storage caused further slight increases.

The only significant ($P < 0.05$) difference in percentage was between dilution and thawing after 24 hours storage.

Variation attributable to dilution rate following the different stages of the freezing process was small, the range being only 0.83 - 2.17% after dilution, 1.67 - 5.25% after equilibration, 5.33 - 7.67% after freezing followed by thawing after 24 hours and 2.83 - 5.00% after freezing followed by thawing after one month's storage.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of acrosomal detachment increased from 2.58 ± 1.248 to 6.08 ± 1.349. There were significant differences ($P < 0.05$) in the cases of samples diluted 1:2, 1:8 and 1:10, in which the means were 5.58, 6.58 and 7.17 respectively.

Analysis of variance of the percentages of spermatozoa with detached acrosomes (table A61) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

1) At the end of equilibration of the diluted semen, there was a marked increase in acrosomal detachment percentage and freezing followed by thawing after 24 hours and one month's storage caused further marked increases. The differences in percentage between stages and also between the two storage times were all significant ($P < 0.05$).

2) The highest detachment percentage was found in samples diluted 1:8 followed by 1:10, 1:4, 1:6, 1:2 and 1:1.

Variation attributable to dilution rate was small, except on thawing after storage for 24 hours, when the percentage of detached acrosomes was significantly higher in samples diluted 1:8 than in those diluted 1:1, 1:6, or 1:10.

The greatest increase in the acrosomal detachment percentages from dilution to equilibration was found in samples diluted 1:10 followed by 1:6, 1:4 or 1:8, 1:2 and 1:1 but the differences were not significant.

The greatest increase from equilibration to thawing after 24 hours storage was found in samples diluted 1:8 followed by 1:4, 1:1, 1:2, 1:6 and 1:10. The difference was significant ($P < 0.05$) in the cases of samples diluted 1:1, 1:4 and 1:8.

The greatest increase from equilibration to thawing after one month's storage was found in samples diluted 1:1 followed by 1:8, 1:4, 1:2, 1:6 and 1:10. The differences were significant ($P < 0.05$) in all cases. There were also significant differences due to storage in the cases of samples 1:1, 1:6 and 1:10.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of acrosomal defects increased slightly from 4.75 ± 1.993 to 7.78 ± 1.262. Differences

were significant ($P < 0.05$) in the cases of samples diluted 1:1, 1:4, 1:8 and 1:10 in which the means were 6.58, 8.50, 7.42 and 8.83 respectively.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A62) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

1) At the end of equilibration of the diluted semen, there was a marked increase in percentage of acrosomal defects and freezing followed by thawing after 24 hours and one month's storage caused further marked increases.

All differences between stage means were significant ($P < 0.05$) but not those between the two storage times.

2) The highest percentage of acrosomal defects was found in samples diluted 1:8 followed by 1:4, 1:10, 1:2, 1:6 and 1:1.

3) Variation attributable to dilution rate at the several stages of the freezing process was small, the ranges being only 6.58 - 8.83% after dilution, 14.75 - 16.67% after equilibration, 23.58 - 30.00% on thawing after 24 hours storage and 29.33 - 32.33% on thawing after one month's storage.

The greatest increase in acrosomal defects from dilution to equilibration was found in samples diluted 1:6 followed by 1:1, 1:8,

1:2 or 1:10 and 1:4. The differences between these stages were significant ($P < 0.05$) in the cases of samples diluted 1:1 and 1:6.

The greatest increase from equilibration to thawing after 24 hours storage was found in samples diluted 1:8 followed by 1:2, 1:4, 1:1, 1:10 and 1:6. The differences between these stages were significant ($P < 0.05$) in the case of samples diluted 1:1, 1:2, 1:4 and 1:8.

The greatest increase from equilibration to thawing after one month's storage was found in samples diluted 1:1 followed by 1:10, 1:8, 1:4, 1:2 and 1:6. The differences between these stages were significant ($P < 0.05$) in all cases.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of tail defect increased very slightly but not significantly from 2.92 ± 0.644 to 3.47 ± 0.265.

Analysis of variance of the percentage of spermatozoa with tail defects (table A63) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to stage of the freezing process.

2) There was no significant difference attributable to dilution rate.

3) There was no significant interaction between the dilution rate and stage.

At the end of equilibration of the diluted semen there was a very slight increase in tail defects.

Freezing of the equilibrated semen followed by thawing after

24 hours and one month's storage caused a slight reduction in tail defects, but the difference was significant ($P<0.05$) at one month only.

Variation attributable to dilution rate was slight except on thawing after 24 hours, when the percentage of tail defects was significantly lower in samples diluted 1:2 than in those diluted 1:8. The latter, however, dropped significantly on thawing after one month.

Figure 43 shows that there was a continuous decrease in percentage of live and motile spermatozoa throughout the freezing process, and the decrease in motility percentage was considerably greater than that of live percentage. There was an associated increase in acrosomal defects.

Figure 44 shows how the different dilution rates affected the percentages of live and motile spermatozoa during the freezing process; least deleterious were dilution 1:1, 1:2 and 1:4 in the case of live percentage and 1:2, 1:4 and 1:6 in the case of motility percentage. In both cases dilution 1:4 was best.

Spermatozoal morphology in general was little affected by dilution rate.

DISCUSSION

The results of this experiment showed that the post thawing live and motility percentages were not greatly affected by dilution rate. However, the post thawing live percentage in the case of 1:8 was inferior to that of 1:4 and to some extent that of 1:6 dilution rates. The highest post thawing motility percentages were found in samples diluted 1:4 or 1:6 and the lowest in samples diluted 1:1.

These findings are very similar to those of experiment No. 3, where glycerol was the cryoprotective, and do not require further discussion.

CONCLUSION

The conclusion of Experiment No. 3, that the optimum rate of dilution for freezing ram semen is 1:4, is confirmed.

13) EXPERIMENT NO. 9

THE EFFECT OF THAWING MEDIUM ON RAM SPERMATOZOA FROZEN IN DIMETHYL SULPHOXIDE DILUENT AND STORED AT -196°C

INTRODUCTION

The aim of this experiment was the same as in experiment No. 4.

MATERIALS AND METHODS

These were the same as in experiment No. 4 except as follows:-

1) The diluent consisted of dimethyl sulphoxide (DMSO) 3%, Lactose (11% solution), 72%, and egg yolk, 25%.

2) The equilibration time was 1.5 hours.

3) Only five of the original six replicates are included in the results, because there was insufficient of one of the samples for evaluation following thawing after one month's storage.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A64 in the appendix.

The analyses of variance of the data for the thawed semen are presented in tables A65-70 in the appendix.

The mean values of the different parameters in the five raw, diluted, and equilibrated semen samples are presented in table 69.

The mean percentages of the different variables in the processed samples on thawing after storage for 24 hours and one month, the standard

errors of their differences and the significant differences at the 5% level are shown in table 70 - live, 71 - motility, 72 - acrosomal damages, 73 - acrosomal detachment, 74 - total acrosomal defects, and 75 - tail defects.

The relationship of the different variables as affected by the stage of the freezing process is illustrated in figure 45 and as affected by thawing medium in figure 46.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa increased slightly, but not significantly from 85.80 $\pm$ 2.800 to 87.10 $\pm$ 1.382.

On equilibration of the diluted semen samples, the mean live percentage fell from 87.10 $\pm$ 1.382 to 73.80 $\pm$ 2.131. This difference was significant ($P < 0.05$).

On freezing of the equilibrated samples followed by thawing with different media after 24 hours and one month's storage, the mean live percentages fell from 73.80 $\pm$ 2.131 to 53.93 $\pm$ 2.736, and 42.29 $\pm$ 2.736 respectively. In both cases, the differences were significant ($P < 0.05$) irrespective of medium.

Analysis of variance of live percentages (table A65) shows the following:-

1) There was a significant difference ($P < 0.05$) attributable to storage time.

2) There was a significant difference ($P < 0.05$) attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

The highest live percentage following 24 hours and one month's storage combined was found in samples thawed with medium No. 7 (no medium) followed by 1, 5, 2, 3, 6 and 4. The difference between samples thawed with media Nos. 7 and 4 was significant ($P < 0.05$).

On freezing followed by thawing, the highest live percentage was found in samples thawed with medium No. 7 followed by 1, 5, 2, 6, 3 and 4 when thawed after storage for 24 hours and by 1, 2, 5, 3, 6 and 4 when thawed after storage for one month. There were significant differences ($P < 0.05$) between samples thawed with media Nos. 1 or 7 and 4 in the case of thawing after one month.

Live percentage in thawed semen was consistently higher after storage for 24 hours than after one month, but the difference was not significant in any individual medium.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly, but not significantly from 86.00 ± 2.449 to 82.00 ± 2.000.

On equilibration of the diluted semen samples, the mean motility percentage fell from 82.00 ± 2.000 to 66.00 ± 2.449. This difference was significant ($P < 0.05$).

On freezing of the equilibrated samples followed by thawing with different media after storage for 24 hours and one month, the mean motility percentage fell from 66.00 ± 2.449 to 21.86 ± 1.804 and 11.57 ± 1.804 respectively. In both cases, the difference was significant ($P < 0.05$) irrespective of medium.

Analysis of variance of percentage of motile spermatozoa (table A66) shows the following:-

1) There was a significant difference ($P < 0.05$) attributable to storage time.

2) There was a significant difference ($P < 0.01$) attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

The highest motility percentage was found in samples thawed with medium No. 7 (no medium) followed by 1, 3, 2, 4 or 6 and 5. The differences between samples thawed with media Nos. 7 and 4, 5 and 6 were significant ($P < 0.05$).

The highest motility percentage was found in samples thawed with medium No. 7 followed by 1, 3, 2, 6, 4 and 5 when thawed after storage for 24 hours and by 1, 3, 5, 2, 4 and 6 when thawed after storage for one month. There were significant differences ($P < 0.05$) between samples thawed in media Nos. 7 and 4 or 5 in case of thawing after 24 hours and between those in Nos. 7 and 4 or 6 in case of thawing after one month.

Motility percentage was consistently higher after storage for 24 hours than for one month, but the difference was significant only in the case of thawing with medium No. 6.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal damage decreased slightly, but not significantly from 1.70 ± 0.406 to 0.40 ± 0.187.

On equilibration of the diluted semen samples the mean percentage of acrosomal damage increased from 0.40 $\pm$ 0.187 to 2.70 $\pm$ 0.682. This difference was significant ($P < 0.05$).

On freezing of the equilibrated samples followed by thawing with different media after storage for 24 hours and one month, the mean percentage of acrosomal damage increased slightly but not significantly from 2.70 $\pm$ 0.682 to 4.07 $\pm$ 1.138 and 3.46 $\pm$ 1.138 respectively.

Analysis of variance of percentage of spermatozoa with damaged acrosomes (table A67) shows the following:-

1) There was no significant difference attributable to storage time.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Variation due to thawing medium following the different storage times was slight, the range being 2.40 - 6.80%, after 24 hours and 2.10 - 4.40% after one month.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal detachment increased from 2.90 $\pm$ 0.797 to 5.30 $\pm$ 1.271. This difference was significant ($P < 0.05$).

On equilibration of the diluted semen, the mean percentage of acrosomal detachment increased further from 5.30 $\pm$ 1.271 to 9.30 $\pm$ 1.393. This difference was significant ($P < 0.05$).

On freezing of the equilibrated samples followed by thawing

with different media, after storage for 24 hours and one month, the mean percentage of acrosomal detachment increased from 9.30 $\pm$ 1.393 to 15.83 $\pm$ 0.351 and 18.26 $\pm$ 0.351 respectively. In both cases the difference was significant ($P < 0.05$) irrespective of medium.

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A68) shows the following:-

1) There was a significant difference ($P < 0.01$) attributable to storage time.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Variation attributable to thawing medium was slight, the range being 12.70 - 17.30% after storage for 24 hours and 16.20 - 19.90% after one month. Detachment percentages were consistently higher after 24 hours storage than after one month but in no single medium was the difference significant.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased slightly but not significantly from 4.60 $\pm$ 1.005 to 5.70 $\pm$ 1.241.

On equilibration of the diluted semen, the mean percentage of acrosomal defects increased markedly from 5.70 $\pm$ 1.241 to 12.00 $\pm$ 1.313. This difference was significant ($P < 0.05$).

On freezing of the equilibrated samples followed by thawing with different media, after storage for 24 hours and one month, the mean

percentage of acrosomal defects increased further from 12.00 $\pm$ 1.313 to 19.90 $\pm$ 0.972 and 21.71 $\pm$ 0.972, respectively. In both cases the differences were significant ($P < 0.05$) irrespective of medium.

Analysis of variance of total percentage of spermatozoa with acrosomal defects (table A69) shows the following:-

1) There was no significant difference attributable to storage time.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Variation attributable to thawing medium was slight, the range being 18.10 - 23.40% after storage for 24 hours, and 20.20 - 24.20% after one month.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of tail defects increased very slightly, but not significantly from 2.00 $\pm$ 0.612 to 2.20 $\pm$ 0.464.

On equilibration of the diluted semen, the mean percentage of tail defects decreased slightly, but not significantly from 2.20 $\pm$ 0.464 to 1.20 $\pm$ 0.406.

On freezing of the equilibrated semen samples followed by thawing with different thawing media after storage for 24 hours and one month the mean percentage of tail defects increased very slightly but not significantly from 1.20 $\pm$ 0.406 to 1.24 $\pm$ 0.311 and 1.67 $\pm$ 0.311 respectively.

Analysis of variance of the percentage of spermatozoa with tail defects (table A70) shows the following:-

1) There was no significant difference attributable to storage time.

2) There was no significant difference attributable to thawing medium.

3) There was no significant interaction between thawing medium and storage time.

Variation attributable to thawing medium was very slight, the range being 0.90 - 1.80% after storage for 24 hours and 1.00 - 2.40% after one month.

Figure 45 shows that from dilution there was a continuous decrease in the percentage of live and especially of motile spermatozoa during the freezing process. There was an associated increase in acrosomal defects and very slight fluctuation in tail defects.

Figure 46 shows the effects of the different thawing media. The percentages of live and motile spermatozoa were highest with media Nos. 7 and 1, but morphology in general was little affected.

DISCUSSION

The results of this experiment indicate that the post thawing live and especially motility percentages were better when the frozen pellet was simply thawed in a dry test tube, than when a thawing medium was used. This might be due to the harmful effect of redilution of the thawed semen, discussed earlier (page 161)and is in agreement with the findings of Salamon (1973), Salamon *et al*. (1973) and Visser (1974a).

Physiological saline was best of the thawing media used here, contrary to experiment No. 4 where glycerol was the cryoprotective used, but again none of the differences attributed to medium was significant.

Results were more consistent here than in experiment No. 4, however, in that in all cases where media were used for thawing they gave slightly better results in a liquid state at 37°C than as frozen pellets at -196°C, and addition of lactoze also gave a slight improvement.

CONCLUSION

Thawing in a dry test tube was found to be the best way of thawing frozen ram semen diluted with DMSO. The result confirms the finding in experiment No. 4, that there is a possibility of disadvantage in increasing the dilution of frozen semen at the time of thawing.

14) EXPERIMENT NO. 10

THE EFFECT OF THAWING TEMPERATURE ON RAM SPERMATOZOA FROZEN IN DIMETHYL SULPHOXIDE DILUENT AND STORED AT -196°C

INTRODUCTION

The aim of this experiment was the same as in experiment No. 5.

MATERIALS AND METHODS

These were the same as in experiment No. 5, except that the six replicates evaluated were the original six frozen in experiment No. 9, in dimethyl sulphoxide (DMSO) diluent.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A71 in the appendix.

The analyses of variance of the data for processed semen are presented in table A72 in the appendix.

The mean values of the different parameters of the six raw, diluted and equilibrated semen samples are presented in table 76.

The mean percentages of the different variables in the processed samples after thawing at the different temperatures, the standard error of their differences and the significant differences at the 5% levels are shown in table 77.

The relationship of the different variables as affected by thawing temperature is illustrated in figure 47.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa increased very slightly, but not significantly from 84.83 $\pm$ 2.482 to 85.92 $\pm$ 1.635.

On equilibration of the diluted semen, the mean percentage of live spermatozoa fell from 85.92 $\pm$ 1.635 to 72.00 $\pm$ 2.503.

On freezing of the equilibrated semen followed by thawing at different temperatures, the mean percentages of live spermatozoa fell further from 72.00 $\pm$ 2.503 to 44.50 $\pm$ 3.980. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of percentage of live spermatozoa (table A72) shows that there was a significant difference ($P < 0.001$) attributable to thawing temperature.

The highest live percentage was found in samples thawed at 60°C followed by 37°C, 100°C, 45°C, 80°C, 20°C and 0°C. The differences between samples thawed at 37°C, 60°C or 100°C and 0°C, and at 20°C and 60°C were significant ($P < 0.05$).

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motility fell slightly, but not significantly from 85.00 $\pm$ 2.236 to 81.67 $\pm$ 1.667.

On equilibration of the diluted semen, the mean percentage of spermatozoal motility fell further from 81.67 $\pm$ 1.667 to 66.67 $\pm$ 2.108. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing at different temperatures, the mean percentages of motility fell markedly from 66.67 $\pm$ 2.108 to 14.77 $\pm$ 3.447. This difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of motile spermatozoa (table A72) shows that there was a significant difference ($P < 0.01$) attributable to thawing temperature.

The highest motility percentage was found in samples thawed at 60°C followed by 100°C, 37°C, 80°C, 45°C, 0°C and 20°C. The difference between samples thawed at 60°C and 20°C was significant ($P < 0.05$).

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of acrosomal damage decreased from 1.92 ± 0.396 to 0.58 ± 0.239. This difference was significant ($P < 0.05$).

On equilibration of the diluted semen, the mean percentage increased from 0.58 ± 0.239 to 3.08 ± 0.676. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen samples followed by thawing at different temperatures, the mean percentages increased further but not significantly from 3.08 ± 0.676 to 9.30 ± 2.551.

Analysis of variance of percentage of spermatozoa with damaged acrosomes (table A72) shows that there was no significant difference attributable to thawing temperature, the range being 6.58 - 13.67%.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of acrosomal detachment increased from 2.50 ± 0.764 to 4.83 ± 1.138. This difference was significant ($P < 0.05$).

On equilibration of the diluted semen, the mean percentage increased further from 4.83 ± 1.138 to 8.50 ± 1.390. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen, followed by thawing at different temperatures, the mean percentage increased from 8.50 ± 1.390 to 16.85 ± 2.256. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A72) shows that there was a significant difference ($P < 0.05$) attributable to thawing temperature.

The highest percentage was found in samples thawed at 20°C followed by 37°C, 60°C, 45°C, 80°C, 100°C and 0°C. The differences between samples thawed at 0° or 100°C and 20°C were significant ($P < 0.05$).

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of acrosomal defects increased slightly but not significantly from 4.42 ± 0.841 to 5.42 ± 1.052.

On equilibration of the diluted semen, the mean percentage increased further from 5.42 ± 1.052 to 11.58 ± 1.150. This difference was significant ($P < 0.05$).

On freezing of the equilibrated semen followed by thawing at different temperatures, the mean percentage increased markedly from 11.58 ± 1.150 to 26.14 ± 2.375. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of percentage of spermatozoa with acrosomal defects (table A72), shows that there was a significant difference ($P < 0.01$) attributable to thawing temperature.

The highest percentage was found in samples thawed at 20°C followed by 80°C, 45°C, 37°C, 0°C 60°C and 100°C. The differences between samples

thawed at 0°C, 60°C or 100°C and 20°C were significant ($P < 0.05$).

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of tail defects increased slightly but not significantly from 1.83 ± 0.527 to 2.33 ± 0.401.

On equilibration of the diluted semen, the mean percentage decreased slightly but not significantly from 2.33 ± 0.401 to 1.25 ± 0.335.

On freezing of the equilibrated semen followed by thawing at different temperatures, the mean percentage decreased very slightly but not significantly from 1.25 ± 0.335 to 1.20 ± 0.392.

Analysis of variance of percentage of spermatozoa with tail defect (table A72) showed that there was no significant difference attributable to thawing temperature, the range being only 0.75 - 1.75%.

Figure 47 shows how the percentages of live and motile spermatozoa were affected by thawing temperature, the least deleterious being in the higher range from 37°C to 100°C and especially 60°C.

Acrosomal integrity was most adversely affected by thawing temperature of 20°C. Tail defects were apparently unaffected by thawing temperature.

DISCUSSION

The results of this experiment indicate that the post thawing live and motility percentages were higher following fast thawing at 37°C - 100°C than those following slow thawing. This is in agreement with the results of experiment 5 where glycerol was used as the cryoprotective.

On the other hand, the percentage of acrosomal defects were higher following thawing at 20°C and 80°C than at other temperatures. This might be due to the possibility of spermatozoa being shocked by exposure during thawing to temperatures higher than of the body (Saacke et al. 1974) or at room temperature (Iype, et al., 1963).

Although rapid thawing at 37°-100°C is clearly the most beneficial with regard to spermatozoal recovery actual spermatozoal temperatures higher than 37°C would be lethal. Moreover the use of most thermometers as well as timing of thawing is subject to human error, e.g. visual error, and the technique cannot be considered sufficiently accurate to adopt high thawing temperatures without risk (Saacke et al., 1974).

CONCLUSION

Although thawing at 37°C resulted in fewer live spermatozoa than at 60°C, and fewer motile spermatozoa and more acrosomal defects than at 60°C or 80°C, none of these differences was significant. Moreover, thawing at 37°C was slightly better than at 60°C in respect of motility and acrosomal defects, but not live percentage in experiment No. 5, where glycerol was used in the diluent.

It is therefore considered best, because of safety, for routine use.

15) EXPERIMENT NO. 11

THE EFFECT OF DIFFERENT CRYOPROTECTIVES ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

The combination of a low level of dimethyl sulphoxide (DMSO) 1.5% along with glycerol, 7%, in a diluent containing a sugar like fructose was superior to the use of DMSO or glycerol alone in storage of ram semen at 4°C (Jones, 1965a) or its freezing (Jones, 1965b). Similarly, Snedeker and Gaunya (1969) found that 2-2.5% DMSO with no more than 5% glycerol gave the highest post freezing motility in bull semen extended in milk diluent.

Porterfield and Ashwood-Smith (1962) found that the combination of DMSO and glycerol, 2.5% of each, or 10% of either separately, was suitable for freezing bone marrow.

The effect of level of glycerol and DMSO on ram semen has been studied in experiments Nos. 1 and 6 respectively. The purpose of this experiment was to compare the effect of each cryoprotective, singly and in combination, and the absence of either cryoprotective upon the viability and morphology of ram spermatozoa and to determine the best cryoprotective for use in the subsequent experiment.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples were divided into five equal volumes in 10 ml tubes. To each tube containing the raw semen one of the following diluents was added directly at a dilution rate of 1:4 (semen:diluent), at 37°C in a water bath.

1. 4% glycerol, 71% lactose (11% solution) and 25% egg yolk.
2. 3% DMSO, 72% lactose (11% solution) and 25% egg yolk.
3. 2% glycerol, 1.5% DMSO, 71.5% lactose (11% solution) and 25% egg yolk.
4. 4% glycerol, 3% DMSO, 68% lactose (11% solution) and 25% egg yolk.
5. 75% lactose (11% solution) and 25% egg yolk.

These dilution rates resulted in final glycerol percentages in diluent No. 1, 3 and 4 of 3.2, 1.6 and 3.2 respectively, and final DMSO percentages in diluent No. 2, 3 and 4 of 2.4, 1.2 and 2.4 respectively. Diluent No. 5 contained no glycerol or DMSO.

The diluted semen samples were equilibrated at $4^{o}C$ for 1.5 hours followed by freezing on dry ice ($-79^{o}C$) and storage in liquid nitrogen ($-196^{o}C$).

Thawing of the frozen pellets was carried out in a dry test tube in a water bath at $37^{o}C$ after 24 hours and one month.

Evaluation of motility was carried out immediately after dilution, at the end of 1.5 hours equilibration, and following thawing after ultra-low temperature storage ($-196^{o}C$) for 24 hours and one month.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentages of live spermatozoa and morphological defects.

Six replicates were evaluated for each diluent.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A73 in the appendix.

The analyses of variance of the data for processed semen are presented in tables A74 - A79 in the appendix.

The mean values of the different parameters in the six raw semen samples are presented in table 78.

The mean percentages of the different variables in the processed samples, the standard errors of their differences and the significant differences at the 5% levels are shown in tables 79 - live, 80 - motility, 81 - acrosomal damages, 82 - acrosomal detachment, 83 - acrosomal defects and 84 - tail defects.

The relationship of the variables as affected by stage of the freezing process is illustrated in figure 48 and as affected by cryo-protective in figure 49.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 89.58 $\pm$ 1.307 to 85.28 $\pm$ 3.710. There was a significant difference ($P < 0.05$) only in case of diluent No. 4 where the mean was 84.33.

Analysis of variance of the percentage of live spermatozoa (table A74) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to the freezing process.

2) There was no significant difference attributable to cryo-protective.

3) There was a significant interaction ($P<0.01$) between cryoprotective and stage.

1) At the end of equilibration of the diluted semen there was a marked reduction in the percentage of live spermatozoa.

On freezing and storage of the equilibrated semen followed by thawing there was a further reduction in live percentages especially after one month's storage.

The differences in percentages between stages of the freezing process were significant ($P<0.05$). The difference attributable to storage time, however, was not significant.

2) Variation between cryoprotectives was slight overall, the range being only 51.42 - 58.29%.

3) Variation between cryoprotectives following the stages of dilution and equilibration was small, the range being only 83.67 - 87.25% and 59.92 - 67.42% respectively.

On thawing, the highest percentage was found in samples diluted with cryoprotective No. 3, followed by 5, 1, 2 and 4 after 24 hours storage, and by 2, 5, 1 and 4 after one month. The differences between samples diluted with cryoprotective No. 3 or 5 and 4 on thawing after 24 hours, and between samples diluted with cryoprotective No. 3 and 4 when thawed after one month, were significant ($P<0.05$).

The greatest drop in live percentage from dilution to equilibration was found in samples diluted with cryoprotective No. 2 followed by 1, 5, 4 and 3. The differences were significant ($P<0.05$) in the cases of cryoprotective No. 1, 2 and 5.

The greatest drop in the percentages from equilibration to

thawing was found in samples diluted with cryoprotective No. 4 followed by 3, 1, 5 and 2 when thawed after 24 hours storage and by 1, 3, 5 and 2 when thawed after one month's storage. The differences were significant ($P < 0.05$) except in the cases of cryoprotectives No. 2 and 5 when thawed after 24 hours storage.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly, but not significantly from 81.67 ± 4.014 to 78.33 ± 2.647.

Analysis of variance of percentage of motile spermatozoa (table A75) showed the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to cryoprotective.

3) There was a significant interaction ($P < 0.001$) between cryoprotective and stage.

1) After equilibration of the diluted semen there was a marked reduction in percentage of motile spermatozoa.

After freezing and thawing there were further reductions expecially after one month's storage.

The differences in percentage between stages of the freezing process and between the two storage times were all significant ($P < 0.05$).

2) The highest motility percentage was found in samples diluted with cryoprotective No. 1, followed by 3, 4, 2 and 5. There were significant differences ($P < 0.05$) between samples diluted with cryoprotective Nos. 1, 2, 3 or 4 and 5 and between samples diluted with

cryoprotective Nos. 1 and 2.

3) Following dilution, motility percentage was the same in all the different cryoprotectives.

Following equilibration, the highest percentage was found in samples diluted with cryoprotective No. 3 followed by Nos. 1, 2 or 4 and 5. There were significant differences between samples diluted with cryoprotective Nos. 1, 2, 3 or 4 and 5.

On thawing the highest percentage was found in samples diluted with cryoprotective No. 1, followed by 4, 3, 2 and 5 after 24 hours storage, and by Nos. 2 or 3, 4 and 5 after one month's storage. There were significant differences ($P < 0.05$) between samples diluted with cryoprotectives Nos. 1, 3 or 4 and 5 and between samples diluted with Nos. 1 and 2 when thawed after 24 hours, and between samples diluted with Nos. 1 and 5 when thawed after one month.

The greatest fall in motility percentage from dilution to equilibration was found in samples diluted with cryoprotective No. 5 followed by 2 or 4, 1 and 3. The difference was significant ($P < 0.05$) in each case.

The greatest drop in motility percentage from equilibration to thawing was found in samples diluted with cryoprotective No. 3 followed by 2, 5, 4 and 1 when thawed after 24 hours storage and by Nos. 4, 2, 1 and 5 when thawed after one month's storage. The differences were significant ($P < 0.05$) in all cases. Moreoever the differences in motility percentage between the two storage times of the frozen semen were significant ($P < 0.05$) in the case of cryoprotective Nos. 1 and 4.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentages of spermatozoal acrosomal damage increased slightly, but not significantly from 1.92 $\pm$ 0.676 to 2.05 $\pm$ 0.721.

Analysis of variance of percentages of spermatozoa with damaged acrosomes (table A76) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to cryoprotective.

3) There was no significant interaction between the cryoprotective and stage. The variation throughout this experiment was very slight and fell with the range 1.42 - 5.33%.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with detached acrosomes increased from 1.50 $\pm$ 0.500 to 3.80 $\pm$ 0.780. The difference was significant ($P < 0.05$) irrespective of cryoprotective.

Analysis of variance of the percentage of spermatozoa with detached acrosomes (table A77) showed the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to cryoprotective.

3) There was no significant interaction between cryoprotective and stage.

1) Following equilibration there was a marked increase in the percentage of detached acrosomes.

After freezing and thawing of the equilibrated semen samples, there was a further increase in the acrosomal detachments, especially when samples were thawed after one month's storage.

There were significant differences ($P < 0.05$) in percentage between all stages of the freezing process but not between storage times.

2) The highest percentage of detached acrosomes was found in samples diluted with cryoprotective No. 4 followed by 5, 2, 3 and 1. The differences between samples diluted with cryoprotective Nos. 4 or 5 and 1 were significant ($P < 0.05$).

3) Variation attributable to cryoprotective following each stage of the freezing process was small, with ranges of only 3.42 - 4.00% after dilution, 8.50 - 12.33% after equilibration, 15.17 - 20.25% after thawing following 24 hours storage and 16.83 - 20.75% after thawing following one month's storage. There was, however, a significant difference ($P < 0.05$) between samples diluted with cryoprotective Nos. 4 and 1 on thawing after 24 hours storage.

The greatest increase in detachments from dilution to equilibration was found in samples diluted with cryoprotective No. 5 followed by 2, 4, 3 and 1. The difference was significant ($P < 0.05$) in all except No. 1.

The greatest increase from equilibration to thawing was found in samples diluted with cryoprotective No. 4 followed by 1, 3, 2 and 5 when thawed after 24 hours storage and by Nos. 3, 5, 1 and 2 when thawed after one month's storage. In all cases the difference was significant ($P < 0.05$).

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased from 3.42 ± 1.158 to 5.85 ± 0.746. The difference was significant ($P < 0.05$) only in the case of cryoprotective No. 4 when the mean was 5.83%.

Analysis of variance of percentage of spermatozoa with acrosomal defects (table A78) showed the following:-

1) There was significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to cryoprotective.

3) There was no significant interaction between cryoprotective and stage.

1) Following equilibration of the diluted semen, there was a marked increase in acrosomal defects.

After freezing and thawing there was a further increase in acrosomal defects especially after one month's storage.

There were significant differences ($P < 0.05$) in percentage between all stages of the freezing process but not between storage times.

2) Variation attributable to cryoprotective was small overall, the range being only 14.67 - 17.67%.

3) Variations attributable to cryoprotective following each stage of the freezing process was small, ranging from 5.42 - 6.25% after dilution, 11.50 - 17.08% after equilibration, 18.75 - 23.00% after freezing followed by thawing after 24 hours storage and 21.17 - 26.08% after freezing followed by thawing after one month's storage. The only significant difference ($P < 0.05$) was between samples diluted with cryoprotectives Nos. 1 and 5 after the stage of equilibration.

The greatest increase in the percentage of acrosomal defects from dilution to equilibration was found in samples diluted with cryoprotective No. 5 followed by 4, 2, 3 and 1. The difference was significant ($P < 0.05$) in all except No. 1.

The greatest increase from equilibration to thawing after 24 hours storage was found in samples diluted with cryoprotective No. 1 followed by 4, 2 or 3 and 5. The difference was significant ($P < 0.05$) in all except No. 5.

The greatest increase from equilibration to thawing after one month's storage was found in samples diluted with cryoprotective No. 3 followed by 2, 1, 5 and 4. The difference was significant ($P < 0.05$) in all cases.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoa with tail defects decreased very slightly but not significantly from 8.83 ± 3.108 to 8.12 ± 0.891.

Analysis of variance of percentage of spermatozoa with tail defects (table A79) showed the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to cryoprotective.

3) There was no significant interaction between the cryoprotective and stage.

Variation throughout this experiment was very small and fell within the range 4.83 - 10.00%.

Figure 48 shows that there was a continuous decrease in the percentages of live and of motile spermatozoa during the freezing process. There was an associated increase in acrosomal defects especially detachments but a very slight change in tail defects.

Figure 49 shows that the percentages of live and motile spermatozoa were affected by the different cryoprotectives, but that those of morphological defects were only slightly affected. Cryoprotective Nos. 3 and 1 were least deleterious to the percentage of live and motile spermatozoa respectively.

DISCUSSION

The result of this experiment indicated that while the best post thawing live percentages were found in samples diluted with a combination of 2% glycerol with 1.5% DMSO, samples diluted with egg yolk and lactose alone appeared to show better spermatozoal survival than samples diluted with 4% glycerol and 3% DMSO separately or in combination.

On the other hand the highest post thawing motility percentages were found in samples diluted with 4% glycerol followed by its combination with DMSO at both levels, 3% DMSO, and egg yolk lactose alone. This indicates that glycerol was better and less toxic as a cryoprotective than DMSO, for the spermatozoa which is in agreement with Jones (1965b) and Malinin et al. (1968) in freezing ram semen, Westfall and Harris (1975) in freezing chicken spermatozoa and Lovelock and Bishop (1959) in freezing bull semen. However, the failure of their combination to produce a better result than glycerol alone was not in agreement with Jones (1965b).

Freezing of ram semen with egg yolk lactose alone produces some protection by attachment of the egg yolk to the spermatozoal membrane (Watson, 1975a, and Watson and Martin, 1975) but the motility results here were very poor. There was a much larger discrepancy between motility and live percentages with this diluent than with those containing glycerol or DMSO, which increase the dead (eosinophilic) count by interaction with the stain as discussed earlier. Acrosomal defects were affected similarly by the different cryoprotectives. However, they were more numerous in the cases of 4% glycerol plus 3% DMSO and egg yolk alone due to higher toxicity of the former and less protection by the latter, as widely recorded in the literature. Tail defects showed slight changes only, irrespective of the cryoprotectives.

A further comparison of the effects on spermatozoal viability and morphology during deep freezing and storage of diluents containing 4% glycerol and 3% DMSO can be made from the results of earlier experiments, where the relevant samples were treated similarly to those in the present experiment.

There were 24 such samples diluted with 4% glycerol comprised of 6 samples in experiment No. 1, equilibrated for 1 hour, and 6 samples in experiments 2, 3, and 4, equilibrated for 3 hours.

There were 18 such samples diluted with 3% DMSO comprised of 6 samples in experiments 7, 9 and 10 all equilibrated for 1.5 hours.

In addition 6 samples in experiment 6 were diluted with 2 and 4% DMSO and equilibrated for 1 hour, with almost identical results, and the mean of these is taken to represent a further 6 samples diluted with 3% DMSO.

There was no evidence in experiment 2 or 7 that there was any

significant effect attributable to equilibration time between 1.0 and 3.0 hours.

All 48 samples in the comparison were diluted 1:4, and thawed without medium added at 60°C.

The means of the percentages of the different variables for the raw and processed semen samples throughout the different stages of the freezing process, the standard errors of their differences and the significant differences (student's t test) at the 5% levels are shown in table 85.

The relationship of the variables as affected by the different stages of the freezing process is illustrated in figure 50.

The comparison showed the following results.

Live spermatozoa

The live percentages in the raw semen used for experiments with DMSO was higher than those used for experiments with glycerol, but the difference was not significant. They remained higher throughout the freezing process, becoming significantly higher ($P < 0.05$) following dilution and following thawing after one month's storage.

Motile spermatozoa

The motility percentage in the raw semen diluted with DMSO was higher than that diluted with glycerol, but the difference was not significant. Following dilution and equilibration motility percentages remained higher with the differences being significant ($P < 0.05$) following dilution.

On freezing, followed by thawing, after 24 hours and one month's storage, however, motility percentages were higher in glycerol than in

DMSO, although the differences were not significant.

Total spermatozoa with acrosomal defects

The total of acrosomal defects in the raw semen diluted with glycerol was higher than in that diluted with DMSO, but the difference was not significant. Total acrosomal defects remained higher throughout the freezing process, the differences being significant ($P < 0.05$) following equilibration and thawing of the frozen semen after one month's storage.

Spermatozoa with tail defects

Variation was similar to that of the total acrosomal defects except that the only significant difference ($P < 0.05$) was that following equilibration.

Figure 50 shows that spermatozoal live percentages remained higher in samples diluted with DMSO than with glycerol throughout the different stages of the freezing process. On the other hand, spermatozoal motility percentages remained higher following dilution and equilibration only, and fell below those in glycerol diluent after freezing. Increase in total acrosomal defects as well as the reduction in the tail defect throughout the freezing process occurred similarly in both DMSO and glycerol diluent in association with reduced spermatozoal viability.

The higher live percentages in samples treated with DMSO is a reflection of their higher level in the raw semen.

The difference in motility percentages between the original raw samples was also maintained to the end of equilibration which is in agreement with the observations of Jones (1965a) on the equal effects

of glycerol and DMSO on spermatozoa stored at 4°C. The higher post thawing motility with glycerol, however, confirms the result of this experiment that glycerol is superior to DMSO for protection during actual freezing of spermatozoa.

Morphological defects were not markedly differently affected by glycerol or DMSO but the percentage of acrosomal defects did tend to increase more with glycerol. Glycerol had this effect according to Jones (1965a & 1972c).

These observations support the evidence of this experiment.

CONCLUSION

The greatest protection to the frozen ram spermatozoa as judged by the post thawing motility was achieved with 4% glycerol.

Exclusion of cryoprotective was markedly deleterious to the spermatozoal motility even during equilibration at 4°C.

No advantage accrued from combinations of DMSO and glycerol.

From the overall comparison between glycerol and DMSO, it is confirmed that while 3% DMSO had little different effect from 4% glycerol during storage at 4°C it gave poorer protection during actual freezing.

16) EXPERIMENT NO. 12

THE EFFECT OF METHOD OF DILUTION ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

Sherman (1963) found that penetration of bull spermatozoa by glycerol was faster at 30°C than at 4°C. Clegg et al. (1965) observed that incubation of bull semen at 32°C with glycerol before freezing increased the penetration of the spermatozoa by glycerol. Quinn et al. (1968a) reported that ram spermatozoa became more resistant to cold shock damage as judged by stain uptake, when incubated at 30°C for at least 30 minutes before cold shock treatment. Pursel et al. (1972a) and Pursel, Johnson and Schulman, (1972) found that incubation of boar semen at 30°C for at least 2.5 hours increased the resistance of the spermatozoa to cold shock. Jones (1972c) found that the addition of glycerol at 5°C increased the rate of acrosomal damage in boar and ram semen. Miller and VanDemark (1954) found that adding the glycerol diluents in equal portions at ten minute intervals, compared with making a complete addition at one time, resulted in slightly higher sperm survival after freezing bull semen.

It can be concluded from these observations that incubation of the diluted semen at 30°C as well as the gradual addition of glycerol should provide some degree of resistance of spermatozoa to the deleterious effects of subsequent cooling or freezing.

The aim of this experiment was to compare the effects of incubating glycerol diluted semen at room temperature (20°C) and at

4°C and of the gradual and instantaneous addition of glycerol on the viability and morphology of ram spermatozoa during the freezing process and storage.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples were divided into four equal volumes in 10 ml tubes. To each tube containing the raw semen, a diluent consisting of 4% glycerol, 71% lactose (11% solution) and 25% egg yolk was added by one of the following methods:

1. Dropping of the diluent against the wall of the tube using a 5 ml syringe with fine needle at 4°C followed by equilibration at 4°C; total time for dilution plus equilibration was 1.5 hours.
2. Dropping of the diluent on the wall of the tubes using a 5 ml syringe with fine needle at 20°C (figure 5) followed by equilibration at 20°C; total time for dilution plus equilibration was 1.5 hours.
3. Direct addition of the diluent at 37°C followed by equilibration at 4°C for 1.5 hours.
4. Direct addition of the diluent at 37°C followed by equilibration at 20°C for 1.5 hours.

The dilution rate in each case was 1:4 (semen:diluent) giving a final glycerol percentage of 3.2. Freezing of the equilibrated semen samples was done on dry ice (-79°C) followed by storage in liquid nitrogen (-196°C).

Thawing of the frozen pellets was done in a dry tube in a water bath at 37°C after 24 hours and one month.

Evaluation of motility was carried out immediately after dilution, at the end of 1.5 hours equilibration, and following thawing after ultra low temperature storage (-196°C) for 24 hours and one month.

Eosin-nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of live spermatozoa and morphological defects. Six replicates were evaluated for each method of dilution.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A80 in the appendix.

The analyses of variance of the data for processed semen are presented in tables A81 - 86 in the appendix.

The mean values of the different parameters of the six raw semen samples are presented in table 86.

The mean percentages of the different variables in the processed samples, the standard errors of their differences and the significant differences at the 5% level are shown in table 87 - live, 88 - motility, 89 - acrosomal damage, 90 - acrosomal detachment, 91 - acrosomal defects, and 92 - tail defects.

The relationship of the different variables as affected by stage of the freezing process is illustrated in figure 51 and as affected by method of dilution in figure 52.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 92.58 $\pm$ 0.978 to 90.15 $\pm$ 2.945. The difference was significant ($P < 0.05$) in case of method No. 1, where the mean was 89.08.

Analysis of variance of percentage of live spermatozoa (table A81) shows the following:

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to method of dilution.

3) There was a significant interaction ($P < 0.001$) between method of dilution and stage.

1) At the end of equilibration of the diluted semen, there was a small, but not significant reduction in live percentage.

On freezing of the equilibrated semen followed by thawing there was a marked reduction in live percentages especially when thawed after one month's storage. The differences in percentages between the stages of equilibration and thawing were significant ($P < 0.05$) but not between the two storage times.

2) The highest live percentage was found in samples diluted by method No. 3 followed by 1, 4 and 2. The differences between No. 1 or 3 and 2 or 4 were significant ($P < 0.05$).

3) Variation attributable to method of dilution was slight, just after dilution, the range being 89.08 - 91.92%.

Following equilibration, however, the highest live percentage was found in samples diluted by method No. 4, followed by 2, 1 and 3, and the differences in percentage between No. 2 or 4 and 3, and between 4 and 1 were significant ($P < 0.05$).

On thawing, the highest live percentage was found in samples diluted by method No. 3 followed by 1, 4 and 2 when thawed after 24 hours storage and by 1, 2 and 4 when thawed after one month's storage. In both cases the differences between 1 or 3 and 2 or 4 were significant ($P < 0.05$).

The greatest drop in live percentages from dilution to equilibration was found in samples diluted by method No. 3 followed by 1, 2 and 4. The difference was significant ($P < 0.05$) only in case of No. 3.

The greatest drop in live percentage from equilibration to thawing after 24 hours storage was found in samples diluted by method No. 2 followed by 4, 1 and 3. The greatest drop from equilibration to thawing after one month's storage was found in samples diluted by method No. 4 followed by 2, 1 and 3. In both cases the differences between stages were significant ($P < 0.05$) irrespective of the method of dilution.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell slightly but not significantly from 86.67 ± 2.108 to 85.00 ± 2.404.

Analysis of variance of percentage of motile spermatozoa (table A82) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to method of dilution.

3) There was a significant interaction ($P < 0.001$) between method of dilution and stage.

1) At the end of equilibration of the diluted semen there was a marked reduction in motility percentage.

On freezing of the equilibrated semen followed by thawing there was a further marked reduction in motility percentage, especially after one month's storage. The differences in percentage between stages, but not storage times, were significant ($P < 0.05$).

2) The highest motility percentage of the freezing process was found in samples diluted by method No. 3 followed by 1, 4 and 2. The differences in percentage between 1 or 3 and 2 or 4 were significant ($P < 0.05$).

3) Following dilution, motility percentage was the same with all methods of dilution.

Following equilibration variation attributable to method of dilution was small, the range being only 65.00 - 74.17%.

On freezing followed by thawing after both 24 hours and one month's storage the highest motility percentages were found in samples diluted by method No. 3 followed by 1, 4 and 2. The differences between 1 or 3 and 2 or 4 were significant ($P < 0.05$).

The greatest drop in motility percentage from dilution to equilibration was found in samples diluted by method No. 1 followed by

2, 4 and 3. The differences were significant ($P < 0.05$) in the cases of Nos. 1 and 2.

The greatest drop from equilibration to thawing after 24 hours storage was found in samples diluted by method No. 2 followed by 4, 1 and 3. The greatest drop from equilibration to thawing after one month's storage was found in samples diluted by method No. 4 followed by 2, 3 and 1. In both cases the differences between stages were significant ($P < 0.05$) irrespective of method of dilution.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with damaged acrosomes increased very slightly, but not significantly from 2.00 ± 0.342 to 2.67 ± 0.883.

Analysis of variance of percentage of spermatozoa with damaged acrosomes (table A83) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

Variation throughout this experiment was very slight, the range of means being only 2.50 - 5.67%.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal detachment increased from 3.17 ± 0.654 to 4.23 ± 1.146. These differences were significant ($P < 0.05$) in the cases of method Nos.

1 and 2 when the means were 4.08% and 4.50% respectively.

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A84) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.01$) attributable to method of dilution.

3) There was a significant interaction ($P < 0.001$) between method of dilution and stage.

1) At the end of equilibration of the diluted semen, there was a marked increase in acrosomal detachment percentage.

On freezing of the equilibrated semen followed by thawing there was a further marked increase in the acrosomal detachment percentage especially when thawed after one month's storage.

The difference in percentage between stages of the freezing process but not between storage times, were significant ($P < 0.05$).

2) The highest acrosomal detachment percentage was found in samples diluted by method No. 4 followed by 2, 1 and 3. The differences between 4 and 1 or 3 were significant ($P < 0.05$).

3) Variation attributable to method of dilution following the stages of dilution and equilibration was small, the ranges being only 4.08 - 4.50% and 9.00 - 12.58% respectively.

On freezing followed by thawing after 24 hours storage the highest acrosomal detachment percentages were found in samples diluted by method No. 4 followed by 2 and 1 or 3. The differences between 4 and 1 or 3 were significant ($P < 0.05$).

On freezing followed by thawing after one month's storage, the

highest percentage was found in samples diluted by method No. 4 followed by 2, 1 and 3. The differences between 2 or 4 and 1 or 3 were significant ($P < 0.05$).

The greatest increase in acrosomal detachment percentage from dilution to equilibration was found in samples diluted by method No. 4 followed by 1, 2 and 3. The difference between these stages was significant ($P < 0.05$) in the cases of Nos. 1, 2 and 4.

The greatest increase in acrosomal detachment percentage from equilibration to thawing both after 24 hours and one month's storage was found in samples diluted by method No. 4 followed by 2, 3 and 1. In both cases the differences were significant ($P < 0.05$) irrespective of method of dilution.

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentages of the spermatozoa with acrosomal defects increased from 5.17 ± 0.813 to 6.90 ± 1.279. The difference was significant ($P < 0.05$) irrespective of the method of dilution.

Analysis of variance of percentage of spermatozoa with acrosomal defects (table A85) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.001$) attributable to method of dilution.

3) There was a significant interaction ($P < 0.01$) between method of dilution and stage.

1) At the end of equilibration of the diluted semen, there was a marked increase in the percentage of acrosomal defects.

On freezing of the equilibrated semen followed by thawing, there was a further marked increase especially after one month's storage.

The differences between stages of the freezing process, but not storage times, were significant ($P < 0.05$).

2) The highest percentage of acrosomal defects was found in samples diluted by method No. 4 followed by 2, 1 and 3. The difference between Nos. 4 and 1 or 3 and between Nos. 2 and 3 were significant ($P < 0.05$).

3) Variation attributable to method of dilution following the stage of dilution was very small, the range being only 6.58 - 7.17%.

Following the stages of equilibration, and freezing followed by thawing after 24 hours and one month's storage, the highest percentages of acrosomal defects were found in samples diluted by method No. 4 followed by 2, 1 and 3. There were significant differences ($P < 0.05$) between Nos. 4 and 3 following equilibration, between Nos. 4 and 1 or 3 and between Nos. 2 and 3 following thawing, both after 24 hours and one month's storage.

The greatest increase in acrosomal defects between stages of the freezing process was found in samples diluted by method No. 4 followed by 2, 1 and 3 from dilution to equilibration, by 2, 3 and 1 from equilibration to thawing after 24 hours storage, and by 1 or 2 and 3 from equilibration to thawing after one month's storage. The differences were significant ($P < 0.05$) irrespective of method of dilution except between dilution and equilibration in the case of method No. 3.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoa with tail defects decreased slightly but not significantly from 6.83 ± 3.190 to 5.08 ± 0.565.

Analysis of variance of percentage of spermatozoa with tail defects (table A86) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was a significant difference ($P < 0.05$) attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

The overall percentage of tail defects was highest in samples diluted by method 2, followed by 1, 4 and 3, the difference between 2 and 3 or 4 being significant ($P < 0.05$). However, variation attributable to the method of dilution following each stage of the freezing process was small and not significant.

Figure 51 shows that there was a continuous decrease in the percentages of live and motile spermatozoa during the freezing process, especially after thawing. There was an associated increase in total acrosomal defects, mainly acrosomal detachment, but only a slight fluctuation in tail defects.

Figure 52 shows how method of dilution affected the live, motility and morphology percentages. Methods Nos. 1 and 3 were least deleterious.

DISCUSSION

The result of this experiment showed that immediately following dilution and equilibration, percentages of live and motile spermatozoa were better in samples diluted and equilibrated at 20°C than in those at 4°C especially when the direct method of dilution was employed. This is in accord with other findings that reduction in the spermatozoal live and motility percentages after storage is greater at 4°C than at 20°C (Watanabe, 1968; Martin and Watson, 1973, and Wilmut et al. 1973). But after freezing and thawing live and motility percentages in samples equilibrated at the generally adopted temperature of 4°C were much higher than those equilibrated at 20°C irrespective of dilution method. In addition the post thawing live motility percentages in samples diluted directly were also better than in those diluted by dropping irrespective of the equilibration temperatures but those differences were not significant.

It seemed that equilibration of samples at 20°C followed by freezing at -79°C on the dry ice produced severe cold shock to the spermatozoa (Mann and Lutwak Mann,1955, and Walton, 1957). Such a sharp drop in temperature would normally kill spermatozoa but in this experiment some were recovered live, therefore the pre-freezing incubation at 20°C appeared to afford resistance to cold shock to some extent as found by Quinn et al.(1968a) and Pursel et al. (1972a & b).

Concerning the morphological changes the percentage of acrosomal defects was especially high in those samples equilibrated at 20°C followed by rapid freezing at -79°C and this is in agreement with Quinn et al. (1969) and Walton (1957) who observed that acrosomal changes increased following rapid cooling and cold shock.

CONCLUSION

Resistance of spermatozoa to freezing was better following dilution and equilibration at 4°C, especially when the direct method of dilution was employed. However, the methods of dilution and equilibration at 20°C although losing an initial advantage in live percentage did give some degree of cold shock resistance to the spermatozoa. Therefore, the effect of a combination of the two methods using slow dilution by dropping for 0.5 hour at 20°C followed by equilibration for 1 hour at 4°C, will be investigated as part of the next experiment.

17) EXPERIMENT NO. 13

THE EFFECT OF EGG YOLK WITH OR WITHOUT SODIUM CITRATE ON RAM SPERMATOZOA DURING DEEP FREEZING AND STORAGE AT -196°C

INTRODUCTION

Glycerol penetration of the spermatozoa of the bull (Sherman, 1963 and Clegg et al., 1965), of the ram (Quinn et al., 1968a; Jones, 1972c) and of boar (Pursel et al., 1972a & b and Jones, 1972c) following incubation of the diluted semen at 30°C before equilibration at 4°C increases the resistance of the spermatozoa against the deleterious effect of cooling and freezing. The addition of the cryoprotectives in three equal parts at ten minute intervals (Miller and Van Demark, 1954 and Sexton, 1975) to the bull or fowl spermatozoa also increased such resistance. The results of the previous experiment showed that dilution and equilibration at 20°C had some initial advantages over the same process at 4°C and subsequently gave some degree of cold shock resistance to the spermatozoa.

The value of the inclusion of egg yolk in diluents for the cooling and deep freezing of ram semen has been recognised for many years. The optimum level of egg yolk may vary depending on the level of glycerol (Saroff and Mixner, 1955 and First et al., 1959a) or other components of the diluents (Salamon and Lightfoot, 1969 and Salamon and Visser, 1972) in freezing ram and bull semen. The quantity of egg yolk necessary to protect ram spermatozoa has been reported as low as 0.75% (Watson and Martin, 1974) and as high as 25% (Fraser, 1968). In addition First et al., (1957) reported that there was no difference between 30, 37.5 and 50% of egg yolk in freezing ram semen. Blackshaw and Emmens (1953) and Emmens and Blackshaw (1955) claimed that 50% egg yolk improved the revival rate of frozen ram spermatozoa.

The inclusion of the sodium citrate in the egg yolk diluent changes the pH value (Salamon, 1968). When the pH of the diluted semen before freezing is higher or lower than the neutral point, the post thawing motility of the ram semen is reduced (Visser, 1969). It can be concluded from these observations that incubation of the diluted semen at 20 or 30°C before equilibration as well as the gradual addition of glycerol should provide some degree of increased resistance of spermatozoa to the deleterious effects of cooling or freezing. At the same time the adjustment of the pH of the diluent around the neutral point and the variation of the egg yolk level might improve their post thawing survival.

The aim of this experiment was to study the effects of 0.5 hour incubation at 20°C (room temperature) as well as egg yolk level, with or without sodium citrate, on the spermatozoa of the ram during the deep freezing process.

MATERIALS AND METHODS

After collection and evaluation, the raw semen samples were divided into five equal volumes in 10ml tubes and diluted and equilibrated as follows:-

1. 4% glycerol + 71% lactose (11% solution) + 25% egg yolk (pH 6.5), added directly at 37°C followed by 1.5 hour's storage at 4°C.
2. 4% glycerol + 71% lactose (11% solution) + 25% egg yolk (pH 6.5) added by dropping at 20°C for 0.5 hour followed by 1 hour storage at 4°C.

3. 4% glycerol + 35.5% sodium citrate (3% solution) + 35.5% lactose (11% solution) + 25% egg yolk (pH 7), added by dropping at 20°C for 0.5 hour followed by 1 hour's storage at 4°C.

4. 4% glycerol + 46% lactose (11% solution) + 50% egg yolk (pH 6.5) added by dropping at 20°C for 0.5 hour followed by 1 hour's storage at 4°C.

5. 4% glycerol + 23% sodium citrate (3% solution) + 23% lactose (11% solution) + 50% egg yolk (pH 7) added by dropping at 20°C for 0.5 hour followed by 1 hour's storage at 4°C.

These five treatments will be referred to as methods of dilution, Numbers 1-5.

The dilution rate in each case was 1:4 (semen:diluent) giving a final glycerol percentage of 3.2. Freezing of the equilibrated semen samples was done on dry ice (-79°C) followed by storage in liquid nitrogen (-196°C).

Thawing of frozen pellets was done in a dry tube in a water bath at 39°C. This thawing temperature was adopted after the mean rectal temperature of 36 normal sheep was found to be 39.3 ± 0.07 (S.D.) Evaluation of motility was carried out immediately after dilution, at the end of equilibration, and following thawing after ultra low temperature storage (-196°C) for 24 hours.

Eosin nigrosin and eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentages of live spermatozoa and morphological defects.

Six replicates were evaluated for each method of dilution.

RESULTS

The counts of the different variables studied in the raw and processed semen samples are shown as percentages in table A87 in the appendix.

The analyses of variance of the data for processed semen are presented in tables A88- A93 in the appendix.

The mean values of the different parameters of the six raw semen samples are presented in table 93.

The mean percentage of the various parameters in the processed samples, the standard errors of their differences and the significant differences at the 5% level are shown in table 94 - live, 95 - motility, 96 - acrosomal damages, 97 - acrosomal detachment, 98 - acrosomal defects and 99 - tail defect.

The relationship of the various parameters as affected by stage of the freezing process is illustrated in figure 53, and as affected by method of dilution in figure 54.

Live spermatozoa

On dilution of the raw semen, the mean percentage of live spermatozoa fell from 88.00 $\pm$ 3.768 to 78.97 $\pm$ 2.012. The difference was significant ($P < 0.05$) irrespective of dilution method.

Analysis of variance of percentage of live spermatozoa (table A88) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between methods of dilution and stage.

There were marked reductions in live percentage at the end of equilibration of the diluted semen and on freezing of the equilibrated semen followed by thawing. The differences were significant ($P < 0.05$) in both cases.

Variations attributable to method of dilution was small, the percentage ranges being only 56.22 - 62.33 over all three stages, 74.42 - 82.83 after dilution, 61.00 - 67.83 after equilibration and 33.25 - 40.17 after thawing.

The greatest drop in live percentage from dilution to equilibration was found in samples diluted by method No. 1 followed by 3, 5, 4 and 2. The differences were significant ($P < 0.05$) in the cases of methods 1, 3 and 5.

The greatest drop in live percentage from equilibration to thawing was found in samples diluted by method No. 4 followed by 5, 2, 3 and 1. The differences were significant ($P < 0.05$) in all cases.

Motile spermatozoa

On dilution of the raw semen, the mean percentage of motile spermatozoa fell from 76.67 ± 3.333 to 68.67 ± 1.386. The difference was significant ($P < 0.05$) only in the case of method No. 5 where the mean was 58.33%.

Analysis of variance of the percentage of motile spermatozoa (table A89) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was a significant difference ($P<0.001$) attributable to method of dilution.

3) There was a significant interaction ($P<0.001$) between method of dilution and stage.

1) There were marked reductions in motility percentage at the end of equilibration of the diluted semen and on freezing of the equilibrated semen, followed by thawing. The differences were significant ($P< 0.05$) in both cases.

2) The highest motility percentage was found in samples diluted by method No. 2 followed by 1, 4, 3 and 5. The differences in percentage between 1, 2 or 4 and 3 or 5 were significant ($P<0.05$).

3) Following dilution the highest percentage was found in samples diluted by method No. 1 or 2 followed by 4, 3 and 5. The differences in percentage between 1 or 2 and 5 were significant ($P<0.05$).

Following equilibration, the highest percentage was found in samples diluted by method No. 4 followed by 2, 1, 5 and 3. The differences between 1, 2 or 4 and 3 or 5 were significant ($P<0.05$).

On thawing, the highest percentage was found in samples diluted by method No. 2 followed by 1, 4, 3 and 5. The differences between 1 or 2 and 3 or 5 and between 2 and 4 were significant ($P<0.05$).

The greatest drop in motility percentage from dilution to equilibration was found in samples diluted by method No. 3 followed by 5, 1, 2 and 4. The differences were significant ($P<0.05$) in the cases of Nos. 1, 3 and 5.

The greatest drop in percentage from equilibration to thawing was found in samples diluted by method No. 4 followed by 2, 1, 5 and 3. The differences were significant ($P<0.05$) in all cases.

Spermatozoa with damaged acrosomes

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal damage increased slightly from 1.08 ± 0.327 to 1.83 ± 0.463. The difference was significant ($P < 0.05$) only in case of method No. 1 when the mean was 2.50%.

Analysis of variance of percentages of spermatozoa with damaged acrosomes (table A90) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

Variation throughout this experiment was very slight, the range of means being only 1.33 - 4.58% overall.

Spermatozoa with detached acrosomes

On dilution of the raw semen, the mean percentage of spermatozoal acrosomal detachment increased from 1.83 ± 1.243 to 3.33 ± 0.591. The difference was significant ($P < 0.05$) irrespective of method of dilution.

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A91) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

1) There were marked increases in acrosomal detachment percentage at the end of equilibration of the diluted semen and on freezing of the equilibrated semen followed by thawing.

The differences in both cases were significant ($P < 0.05$).

2) The highest acrosomal detachment percentage was found in samples diluted by method no. 4 followed by 3, 5, 1 and 2. The difference in percentage between nos. 4 and 2 was significant.

3) Variation attributable to method of dilution following the stages of dilution and equilibration was small, the ranges of percentage being only 2.92 - 3.75 and 7.83 - 9.58 respectively.

On thawing the highest percentage was found in samples diluted by method no. 4 followed by 5, 3, 1 and 2. The differences between No. 4 or 5 and 2 were significant ($P < 0.05$).

The greatest increase in acrosomal detachment percentage from dilution to equilibration was found in samples diluted by method no. 4 followed by 1, 3 or 5 and 2.

The greatest increase in percentage from equilibration to thawing was found in samples diluted by method nos. 1, 4 or 5 followed by 3 and 2.

In all cases the differences in percentage between stages were significant ($P < 0.05$).

Total spermatozoa with acrosomal defects

On dilution of the raw semen, the mean percentage of spermatozoa with acrosomal defects increased from 2.92 ± 1.535 to 5.17 ± 0.407. The difference was significant ($P < 0.05$) irrespective of method of dilution.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A92) shows the following:-

1) There was a significant difference ($P < 0.001$) attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

There were marked increases in the percentage of acrosomal defects at the end of equilibration of the diluted semen and on freezing of the equilibrated semen, followed by thawing. The differences were significant ($P < 0.05$) in both cases.

Variation attributable to dilution method was slight, the range in percentage being only 11.11 - 12.47 over all three stages combined, 4.75 - 5.42 after dilution, 10.92 - 12.08 after equilibration, and 17.17 - 19.92 after thawing.

The greatest increase in the total acrosomal defect percentages from the stage of dilution to the stage of equilibration was found in method no. 5 followed by 1, 4, 3 and 2.

The greatest increase in the total acrosomal defect percentages from the stage of equilibration to the stage of freezing followed by thawing after 24 hour's storage was found in method no. 3 followed by 1, 4, 5 and 2.

In all cases the differences between the stages were significant ($P < 0.05$) irrespective of the method of dilution.

Spermatozoa with tail defects

On dilution of the raw semen, the mean percentage of spermatozoa with tail defects increased from 4.58 $\pm$ 1.060 to 8.18 $\pm$ 1.024. There were significant increases ($P < 0.05$) in the cases of samples diluted by method nos. 1 and 2 when the means were 8.83% and 14.08% respectively.

Analysis of variance of percentage of spermatozoa with tail defects (table A93) shows the following:-

1) There was no significant difference attributable to stage of the freezing process.

2) There was no significant difference attributable to method of dilution.

3) There was no significant interaction between method of dilution and stage.

Variation attributable to method of dilution was small throughout this experiment, the overall range being only 4.17 - 9.33 except for samples diluted by method no. 2, where after dilution only the percentage of tail defects at 14.08 was significantly higher ($P < 0.05$) than in samples diluted by methods 3, 4 and 5.

Figure 53 shows that there was a continuous decrease in the percentages of live and especially motility percentages during the freezing process. There was an associated increase in acrosomal defects and slight fluctuation in tail defects.

Figure 54 shows that the method of dilution had a marked effect on the motility percentages, but live and morphology percentages were much less affected. Methods No. 1, 2 and 4 were least deleterious, No. 2 being superior to all.

DISCUSSION

The result of this experiment indicated that the post thawing live percentages were not significantly affected by the different diluents, or by the different methods of dilution. On the other hand significantly higher post-thawing motility percentages were found in samples diluted with standard diluent (4% glycerol, 25% egg yolk and 71% of 11% lactose), especially when added slowly by dropping, than with the other diluents especially when they contained sodium citrate.

Slow addition of the standard diluent by dropping resulted in a post thawing motility of 33.3% compared with 27.5% following direct dilution, but the difference was not significant. Slow addition of the diluent at the room temperature might hasten the glycerol penetration (Sherman, 1963 and Clegg et al., 1965), which in turn increases the resistance of the spermatozoa against cold shock as observed by Quinn et al. (1968a) and Pursel et al. (1972 a & b). The post thawing motility in samples slowly diluted with 25% egg yolk (Method No. 2 and 3) was better than in those diluted with 50% egg yolk (Methods 4 and 5) the means being 20 and 10 per cent respectively, which is in disagreement with the findings of Blackshaw and Emmens (1953) and Emmens and Blackshaw (1955).

This effect might be due to binding of glycerol by the high level of egg yolk as found by Saroff and Mixner (1955); First et al. (1961a) and Malinin et al. (1968). The inclusion of the high level of egg yolk also led to increased acrosomal detachment which is similar to the findings of Pursel, Schulman and Johnson (1973) that increasing egg level from 0 to 30 per cent increased acrosomal defects in boar semen following cold shock.

The inclusion of sodium citrate was markedly deleterious to post thawing motility. The mean percentage of 24.2 for methods 2 and 4 combined was significantly higher than the mean of 5.9 for methods 3 and 5.

Therefore it is probable that the inclusion of sodium citrate in the diluent produced an alteration in pH (Salamon, 1968) which in turn reduced the pre-freezing (Lapwood and Martin, 1972), as well as the post thawing motility (Visser, 1969 and Visser and Salamon, 1974).

Moreover post thawing motility fell with level of lactose (11% solution), the motility being highest with 71% (method no. 2) followed by 46% (method no. 4), 35% (method no. 3) and 23% (method no. 5). Reduced lactose might be a deleterious factor additional to the higher level of egg yolk or the presence of citrate. Lactose is an active sugar in protecting ram spermatozoa when used around 75% (11% solution) according to Fraser (1968); Sainsbury (1968); Aamdal and Andersen (1968b); Salamon and Lightfoot (1969) and Andersen and Aamdal (1972).

CONCLUSION

Dilution by dropping for 0.5 hour at 20°C (room temperature), followed by 1 hour storage at 4°C did not give very different results from the direct methods of dilution at 37°C followed by 1.5 hours equilibration at 4°C. The latter method, although slightly inferior with respect to recovery of motile spermatozoa after freezing and thawing, is simpler and more convenient to perform.

There were unsatisfactory results following inclusion of sodium citrate, increasing the egg yolk level over 25% or reduction of the lactose level from 71%.

18) EXPERIMENT NO. 14

THE EFFECT OF THAWING TEMPERATURE PLUS SUBSEQUENT INCUBATION AT 39°C ON FROZEN RAM SPERMATOZOA STORED AT -196°C

INTRODUCTION

Incubation around body temperature following freezing and thawing has been commonly used to test the efficiency of some factors involved in the freezing process, (Robbins et al., 1972) such as prefreezing treatments or different thawing media after thawing but the most relevant was to measure the longevity of the thawed spermatozoa in vitro as a criterion of potential fertility.

Salamon (1973) claimed that the duration of spermatozoal viability following freezing and thawing could be considered as an important criterion of the functional integrity of boar spermatozoa.

Coulter and Foot (1974) claimed that a reduction in spermatozoal viability and an increase in acrosomal damage of frozen thawed bull spermatozoa was associated with the length of the incubation - the longer the period the faster the reduction in spermatozoal motility and the greater the damage to the acrosome. Similarly, in the ram Quinn and White (1968); Quinn et al. (1969) and Visser (1974a) showed a continuous reduction in spermatozoal motility associated with a continuous increase in acrosomal defects.

From previous experiments (nos. 5 and 10) it was found that fast thawing i.e. at a temperature above 37°C, was better than slow thawing. Almquist and Wiggin (1973a & b) also found that fast thawing at high temperatures (35°C - 95°C) was better than slow thawing at lower temperatures. On the contrary Zakrzewska (1962) found that thawing at room temperature (20°C) and 40°C made no difference to immediate post-

thawing motility. After 0.5 hour incubation motility was higher in samples thawed at room temperature.

The aim of this experiment was to compare the effect of various thawing temperatures on frozen ram spermatozoa as judged by their motility and morphological acrosomal defects after incubation at 39°C. This incubation temperature was chosen because the mean body temperature of 36 healthy sheep was found to be 39.29 ± 0.071°C.

MATERIALS AND METHODS

Part of the frozen semen, diluted by method no. 2 in the previous experiment (No. 13) was used in this experiment. After storage for 24 hours in liquid nitrogen (-196°C) the frozen pellets were thawed at 0°C, 20°C, 39°C, 60°C and 100°C, followed by incubation at 39°C in a water bath.

Evaluation of motility was carried out immediately after each thawing (0 hour incubation) and every three hours during incubation. Eosin fast green FCF stained smears were prepared at the same time for subsequent evaluation of percentage of acrosomal defects.

Six replicates were evaluated for each thawing temperature.

RESULTS

The counts of the different variables studied in the raw, diluted, equilibrated and thawed and incubated semen samples are shown as percentages in table A94 in the appendix. In this experiment, however the percentage of motile spermatozoa after 6 hour's incubation was zero except in two samples where it was very low. Evaluation of acrosomal morphology was therefore omitted at this stage.

The analyses of variance of the data for thawed and incubated semen at 0 and 3 hours are presented in tables A95-98 in the appendix. The mean values of the different parameters in the six equilibrated semen samples are presented as percentages in table 100.

The mean percentages of the different variables in the samples at the different thawing temperatures and incubation times, the standard error of their differences, and the significant differences at the 5% levels are shown in tables 101 - motility, 102 - acrosomal damage, 103 - acrosomal detachment and 104 - acrosomal defects.

The relationship of the different variables (motility and acrosome morphology) as affected by incubation time is illustrated in figure 55 and as affected by thawing temperature in figure 56.

Motile spermatozoa

On freezing of the equilibrated semen followed by 24 hour's storage and thawing at different temperatures, the mean percentage of motile spermatozoa fell markedly from 61.67 ± 7.032 to 24.33 ± 2.952. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of motile spermatozoa (table A95) showed the following:-

1) There was a significant difference ($P < 0.01$) attributable to incubation time.

2) There was a significant difference ($P < 0.001$) attributable to temperature.

3) There was a significant interaction ($P < 0.001$) between thawing temperature and incubation.

Following 3 hours incubation there was a marked reduction in motility percentage which was zero in the majority of samples.

The highest mean percentage of motile spermatozoa over both incubation times was found in samples thawed at 39°C followed by 100°C, 60°C, 20°C and 0°C. The differences in percentage between 39°C, 60°C or 100°C and 0°C or 20°C were significant ($P < 0.05$).

Immediately after thawing the highest percentage was found in samples thawed at 100°C followed by 39°C, 60°C, 20°C and 0°C. The differences in percentage between 39°C, 60°C or 100°C and 0°C or 20°C were significant ($P < 0.05$).

Following incubation for three hours, the highest percentage was found in samples thawed at 39°C followed by 60°C, 100°C and 20°C with no motility in case of 0°C, but motility percentage was low in all samples and there were no significant differences between means.

The greatest drop in motility percentages during the three hour incubation was found in samples thawed at 100°C followed by 60°C or 39°C, 20°C and 0°C. The differences were significant ($P < 0.05$) in case of 39°C, 60°C and 100°C. After six hours incubation, a few motile spermatozoa were seen in two samples only, both thawed at 39°C.

Spermatozoa with damaged acrosomes

On freezing of the equilibrated semen followed by 24 hour's storage and thawing at different thawing temperatures, the mean percentages of spermatozoa with acrosomal damage increased from 3.08 ± 0.831 to 6.42 ± 0.992. There was a significant difference ($P < 0.05$) only in the case of thawing at 60°C when the mean was 6.75%.

Analysis of variance of the percentage of spermatozoa with damaged acrosomes (table A96) shows the following:-

1) There was no significant difference attributable to incubation time.

2) There was no significant difference attributable to thawing temperature.

3) There was no significant interaction between the thawing temperature and the incubation time.

Variation due to thawing temperature throughout the two incubation times was small, the range being only 3.83 - 8.83% overall.

Spermatozoa with detached acrosomes

On freezing of the equilibrated semen followed by 24 hour's storage and thawing at different thawing temperatures, the mean percentage of the spermatozoal acrosomal detachment increased from 7.83 ± 2.386 to 14.73 ± 0.480. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of percentage of spermatozoa with detached acrosomes (table A97) showed the following:-

1) There was a significant difference ($P < 0.001$) attributable to incubation time.

2) There was no significant difference attributable to thawing temperature.

3) There was no significant interaction between thawing temperatures and incubation time.

Following 3 hour's incubation there was a marked increase in acrosomal detachment percentage.

Variation due to thawing temperature was small, the ranges being only 12.75 - 16.67% and 19.17 - 22.17%, after incubation for 0 and 3 hours respectively. The greatest increase in acrosomal detachment percentages during the 3 hour incubation was found in samples thawed at $0^{o}C$ or $39^{o}C$ followed by $60^{o}C$, $100^{o}C$ and $20^{o}C$. The increases were significant ($P < 0.05$) in all cases except thawing at $20^{o}C$.

Total spermatozoa with acrosomal defects

On freezing of the equilibrated semen followed by 24 hour's storage and thawing at different temperatures, the mean percentage of spermatozoa with acrosomal defects increased from 10.92 ± 1.938 to 21.15 ± 1.272. The difference was significant ($P < 0.05$) irrespective of thawing temperature.

Analysis of variance of the percentage of spermatozoa with acrosomal defects (table A98) showed the following:-

1) There was a significant difference ($P < 0.05$) attributable to incubation time.

2) There was a significant difference ($P < 0.01$) attributable to thawing temperature.

3) There was no significant interaction between thawing temperature and incubation time.

Following 3 hour's incubation there was an increase in acrosomal defects.

The highest mean percentage of acrosomal defects over both incubation times, was found in samples thawed at $100^{o}C$ followed by $20^{o}C$, $60^{o}C$, $0^{o}C$ and $39^{o}C$. The difference in percentage between $100^{o}C$ and $39^{o}C$ was significant ($P < 0.05$).

Immediately after thawing the highest percentage was found in samples thawed at 100°C followed by 60°C, 20°C, 0°C and 39°C. The difference in percentage between 100°C and 39°C was significant ($P < 0.05$).

Following incubation for 3 hours the highest percentage was found in samples thawed at 100°C followed by 20°C, 60°C, 0°C and 39°C. The differences in percentage between 100°C and 0°C or 39°C were significant ($P < 0.05$).

The greatest increase in percentage of acrosomal defects during the 3 hour incubation was found in samples thawed at 39°C followed by 20°C, 100°C, 60°C and 0°C. The increases were significant ($P < 0.05$) only in the cases of 20°C and 39°C.

Figure 55 shows that there was a continuous decrease in motility percentages during the freezing process as well as during incubation at 39°C. There was an associated increase in acrosomal defects.

Figure 56 shows that motility and acrosomal defects were affected by the different thawing temperatures, the least deleterious for motility being in the high range (39°C, 60°C and 100°C). The least deleterious for acrosomal defects was 39°C.

DISCUSSION

The result of this experiment indicated that the post-thawing motility of the frozen semen was better when thawed rapidly at 39°C to 100°C than slowly at 20 or 0°C. Such findings were in agreement with those of experiments no. 5 and 10.

However, motility was still superior after three hour's incubation at 39°C and this is contrary to the findings of Zakrzewska (1962).

Generally post thawing spermatozoal motility of mammalian spermatozoa declines following incubation as a result of an intracellular accumulation of toxic substances along with loss of vital proteins such as DNA. The reduction in motility of ram spermatozoa following three hour's incubation at 39°C after thawing was in agreement with findings of Quinn and White (1968) and Quinn et al. (1969).

The increase in acrosomal defects and their association with continuous reduction in the spermatozoal motility following the incubation at 39°C for three hours were in agreement with the results of Quinn and White (1968); Quinn et al. (1969); Visser (1974a) and Coulter and Foot (1974).

The absence of motile spermatozoa following six hour's incubation, except for a very few in two samples only, indicates that their remaining life span after freezing and thawing is very short. It is unlikely that frozen ram semen can be used successfully for artificial insemination other than within a few hours prior to ovulation.

CONCLUSION

Rapid thawing is clearly the most beneficial with regard to spermatozoal recovery as found in Experiment No. 5 and 10. Moreover the thawing temperature of 39°C was least deleterious to both spermatozoal motility and acrosomal morphology here and it avoids all risk of high temperature shock.

It is also evident that ram spermatozoa after freezing and thawing have a short life span at body temperature of around three hours.

19) EXPERIMENT NO. 15

SPERMATOZOAL MORPHOLOGY UNDER THE ELECTRON MICROSCOPE DURING DEEP FREEZING AND STORAGE AT -196°C.

INTRODUCTION

Since low temperature storage of ram spermatozoa was first described by Emmens and Blackshaw (1950), many research workers have demonstrated that while motility can be preserved satisfactorily especially with diluents containing glycerol, it is usually associated with very low fertility as reported in the reviews of Emmens and Blackshaw (1956); Emmens and Robinson (1962); Lunca (1964); Nishikawa (1964 & 1972) and Visser (1974c).

Marked damage to the plasma and acrosomal membranes of the ram spermatozoa following cooling and freezing treatment as observed by Quinn et al. (1969); Healey (1969); Nath (1972) and Watson (1975b) could be an important causal factor of low fertility.

The purpose of this study was to determine the ultra-structural changes in the spermatozoa of raw and diluted ram semen during the different stages of the freezing process which could not be revealed under the light microscope.

MATERIALS AND METHODS

Samples of raw semen and diluted semen just after dilution, equilibration at 4°C for 1.5 hours and thawed after 24 hour's storage in liquid nitrogen (-196°C) were studied by electron microscopy following methods similar to those of Pedersen (1970).

Aliquots were pipetted into 2.5% glutaraldehyde in 1ml s - collidine buffer and allowed to fix for 15 minutes. The suspension was then centrifuged (1500 x g) for ten minutes. The supernatant was drained off and 1ml s - collidine added gently and left for 15 minutes to rinse the pellet. Then the buffer was poured off and one per cent osmium tetroxide (in the same buffer) was added and left for one hour. The pellet was subsequently dehydrated in a graded series of ethanol solution and embedded in Epon 812.

For light microscopy, thin sections were cut on glass knives, stained with toluidine blue and examined for the exact location of the spermatozoa.

Thin sections were cut on a diamond knife, picked up on coated grids, stained successively with uranyl acetate and lead citrate and examined in an A.E.I. 6B Electron-Microscope.

RESULTS & OBSERVATIONS

Ultrastructure of spermatozoa in raw and processed semen samples studied under the electron microscope is summarized and illustrated in the following figures:

I Acrosome

a) Intact acrosome (figure 57)

b) Acrosomes with different forms of defects (figure 59-68)

1. Defect of the plasma membrane (figure 59 & 60)
2. Fractured outer acrosomal membrane (figure 59 & 60)
3. Fractured acrosome (figure 61)
4. Vacuole formation in the acrosomal matrix (figure 60 to 63)

5. Release of the acrosomal content (figure 64 to 66)

6. Defects of the inner acrosomal membrane and of the sub-acrosomal space (figures 64, 66 & 67)

7. Detached acrosome (either separated but still lying in front of the nucleus or completely lost) (figures 66 to 68)

II Post nucleur cap (PNC) (figure 69)

a) Intact PNC (figure 69)

b) Damaged PNC (figure 69)

III Tail

a) Normal cross sections of the midpiece, main piece and end piece (figure 58)

b) Defect of the midpiece (figure 70)

c) Defects of the tail at different levels can be observed in figures 59, 60, 62, 66, 68, 69 & 70.

DISCUSSION

The structural morphology of the acrosome (figure 57), post nucleur cap (figure 69) and tail (figure 58) as revealed by the electron microscope did not differ from what is already described in the Review of the Literature under the heading of "normal spermatozoal morphology", and in the review on mammalian spermatozoa by Fawcett (1975). Sagittal sections through the intact acrosome (figure 57) and tails (figure 58) showed that their plasma membranes were smooth and their matrixes dense which is in agreement with Healey (1969) and Yasuda and Tanimura (1974), but occasionally the plasma membrane, especially around the acrosome, showed distortion which might have arisen during fixation as an artefact

as reported by Jones (1971b, 1972c & 1973c).

On the other hand, different forms of damage to the acrosome ended in the complete detachment of the acrosome leaving the nucleus just covered by the inner acrosomal membrane and even the latter sometimes disintegrated and disappeared. However, in most of the literature so far studied it was claimed that acrosomal defects or changes started by the disruption of the plasma membrane (Zamboni and Stephanini, 1968) followed by swelling and reduction in the acrosomal density (Quinn et al., 1969).

Acrosomal damage started with the distortion of the outer acrosomal membrane followed by its rupture and the release of the acrosomal content leaving the nucleus either surrounded by the empty acrosome or completely uncovered. Such a series of changes was termed a non-specific acrosomal reaction (Boender, 1968; Saake and Marshall, 1968; Williamson, 1974a & b, and Wooding, 1975) which was similar to those of the true (specific) reactions which occurred during capacitation and fertilization (Jones, 1972a and Austin, 1972 & 1975). The series of the acrosomal changes as illustrated in figures 59-68 were quite similar to those observed following cooling and freezing of ram semen by Healey (1969); Quinn et al. (1969); Nath (1972) and Watson (1975b).

In the tail especially the midpiece which was the site of the freezing damage following cooling and freezing, the matrix of the mitochondria became lighter followed by breakage of the plasma membrane (Quinn et al., 1969) and in some cases the fibres shifted to one side which might be due to the presence of ice crystals on the other side (Nath, 1972). Such changes in the midpiece were also observed in human spermatozoa following freezing (Pedersen and Lebech, 1971;

Pedersen, 1972; Leverage et al., 1972) as well as in boar spermatozoa (Jones, 1972a).

CONCLUSION

The ultra-structural changes have been observed in the raw as well as the processed semen at the different stages of the freezing process especially after thawing. The changes included morphological changes of the acrosome as well as the tail especially the midpiece.

20) EXPERIMENT NO. 16

THE EFFECT OF PROMPT DILUTION AFTER COLLECTION AND PREVENTION OF TEMPERATURE FLUCTUATIONS AFTER INITIAL FREEZING ON THE PERCENTAGE OF MOTILE SPERMATOZOA IN RAM SEMEN, AND AN ASSESSMENT OF THE FERTILITY OF THE SEMEN FROZEN BY THIS METHOD IN AN INSEMINATION TRIAL.

INTRODUCTION

The results of the foregoing experiments on the effects of deep freezing on the spermatozoa of the ram have consistently shown that there is a progressive deterioration in semen quality at each stage of the freezing process and during storage at -196°C. The percentage of live and motile spermatozoa decrease and the percentage of acrosomal defects increases.

The effect on motility, a widely accepted criterion for assessment of potential feasibility, is particularly severe. The difference in motility percentages between successive stages and between storage times is statistically significant in nearly all cases.

It is probable that some avoidable loss in motility is attributable to the deleterious effects of environmental factors, particularly temperature, during handling of the semen samples. This might occur during the delay before dilution, while the sample is being transported to the laboratory and evaluated. Willet et al. (1940) found that short storage of undiluted bull semen at 37°C before dilution had a deleterious effect on the motility of spermatozoa after dilution.

It is also possible for temperature fluctuations to occur when frozen pellets are exposed to air temperature (20°C) during transfer from the dry ice block to the liquid nitrogen storage and during the addition of fresh batches of pellets to the store or removal of samples of pellets

from the store, especially if different samples have to be stored on the same carrier cane (figure 8). Fraser (1970) claimed that frozen pellets of ram semen lost their fertility if exposed even briefly to air temperature on three or more occasions. Krause (1972) claimed that variation in temperature of frozen semen after long-term storage and transportation had to be avoided, otherwise changes in crystallisation might occur below -80°C and spermatozoa might be damaged.

Generally the fertility of frozen ram semen has been poor since low temperature storage was first described by Emmens and Blackshaw (1950). On the other hand, high conception rates have been obtained by a few investigators, but mostly either by using more than one insemination during a single oestrus (First et al., 1961b; Kareta, et al., 1971 & 1972a and Brice, 1972) or by intrauterine or intratubal inseminations following laparotormy (Fraser, 1968; Mattner, Entwistle and Martin, 1969; Loginova and Zheltobryukh, 1972a & b and 1974; Andersen et al., 1973, and Gustafsson, 1975).

Fertility was enhanced by the addition of prostaglandin to the thawed semen according to Gustafsson and Linge (1975) and Gustafsson, Edqvist, Einarsson and Linge (1975).

However, in the ram, contrary to the bull, fertility of frozen semen never approaches that of fresh semen (Nishikawa, 1972).

The aim of this experiment was to study the effects on the viability of spermatozoa, as assessed by motility, of some modifications of the freezing process designed to overcome such possible deleterious influences. A small artificial insemination trial was also planned to test the fertility of the semen frozen by this method.

MATERIALS AND METHODS

Dilution and equilibration

Immediately after collection the semen was diluted directly 1:4 with 4% glycerol, 25% egg yolk and 71% of 11% lactose solution, previously warmed to 37°C, transferred at once to the laboratory in a thermos flask at 37°C, and equilibrated at 4°C for 1.5 hours.

Freezing

At the end of equilibration, the diluted semen was frozen in pellets on dry ice in the usual way. When the pellets solidified they were dropped into a dish filled with liquid nitrogen (figure 71). Then, after 1-2 minutes the excess liquid nitrogen was poured off and the rest containing the frozen pellets was poured into a plastic tube, previously cooled to -196°C (figure 72), which was then lowered into a liquid nitrogen flask for further storage. The tube was suspended in the upright position on a length of string, with an identification label attached to the proximal end.

Sampling

To obtain a sample for thawing, the required tube was raised to the mouth of the storage flask, and 2 or 3 frozen pellets were removed using an aluminium spoon (figure 73). The remaining pellets, still immersed in liquid nitrogen in the tube, were then returned below the surface of the nitrogen in the flask. Thawing was carried out in a dry test-tube at 39°C.

Evaluation of motility of each sample was made after dilution and return to the laboratory, after equilibration, and following thawing

after ultra low temperature storage at -196°C for 24 hours (T1) and 42-46 days (T2).

Five replicates were evaluated.

Artificial insemination trial

The ewes for insemination were part of a late lambing flock of about 100 mainly half-bred ewes due to be mated from mid November. In order to avoid a protracted lambing artificial insemination was restricted to those ewes in heat during the 5 days commencing 11th November 1974. A raddled vascetomised ram was run with the flock, which was inspected night and morning, and any marked ewe withdrawn for insemination 24 hours later.

Semen for the insemination was thawed in the laboratory just before required, examined for motility and if satisfactory transported to the ewe in a flask at 39°C. The first thawing and insemination were carried out 42 days after initial freezing.

Two inseminations, 12 hours apart, were performed on each ewe, using a dose of 0.1-0.2 ml. of thawed semen deposited on the cervical os with the aid of a vaginal speculum.

A total of 28 ewes were inseminated.

RESULTS

The percentages of motile spermatozoa immediately after dilution after equilibration for 1.5 hours, and after freezing followed by thawing after 24 hours and 42 day storage are presented in table 105.

Analysis of variance of the motility percentages, presented in Appendix table No. A99, showed that there was a significant difference between stages of the freezing process.

Comparison of the means in table 105 shows that there was no significant change in motility between the stages of dilution and equilibration. There were however, significant reductions ($P < 0.05$) between equilibration and thawing after freezing, and between the two storage times. The last reduction was particularly severe, being equivalent to a loss of 50% or more of the motile spermatozoa present after 24 hours storage in all 5 samples.

All the samples from the 2 Dorset rams were discarded, and only the samples of the 3 Suffolk rams (30-40% motility) were used for the insemination trial.

Despite this restriction, the conception rate was zero, all 28 ewes returning to oestrus.

DISCUSSION

The modified methods of handling the semen samples introduced in the present experiment resulted in the highest recovery of motile spermatozoa after 24 hours storage at -196°C, so far in this study, but the improvement was not maintained over more prolonged storage.

It was not possible to run a direct control using standard technique, because, the time required to pellet and store one batch of semen would have meant either excessively delayed dilution or prolonged equilibration of the other, before it could be pelleted and stored. The results of the modified technique can, however, be compared with the results of previous experiments, viz. Method 3 in Experiment No. 12 and Method 1 in Experiment No. 13, where the same diluent, dilution rate, and equilibration time were employed. These are set out in table 106 and their analysis of variance is presented in Appendix table A100.

There was little difference in motility between the two groups of samples after dilution, but the modified method led to a reduced loss of motility after equilibration, so that the difference between these two stages was no longer significant. It did not prevent the usual significant reduction from equilibration to freezing and thawing after 24 hours. Nevertheless, at this stage there were still 59.0% motile spermatozoa compared with 37.1% using the standard method. Students t-test shows this to be a significant difference ($P < 0.05$). After storage for 42 days or more, however, motility was again almost identical with earlier findings after one month's storage.

Prompt dilution of the ram semen samples was claimed by Polge (1974) to improve the resistance of spermatozoa to harmful environmental effects. Here this led to an improvement following cooling and equilibration, which is in agreement with Willet et al. (1940).

The elimination of the risk of possibly harmful temperature fluctuations (Fraser, 1970) during transfer of the frozen pellets from the dry ice blocks to the storage tank of liquid nitrogen also appeared to be effective, as indicated by the satisfactory motility after 24 hour's storage, although this must be partially due to the reduced damage in the earlier stages. On the other hand the marked reduction in spermatozoal motility following the storage of the frozen pellets from 42-46 days in the liquid nitrogen, in spite of the new sampling technique employed to eliminate temperature rises above -196°C, indicates that storage itself causes such reduction. This result is in agreement with those throughout this study and of Hill et al. (1959) and First et al. (1961a), who observed a sharp drop in post thawing motility of frozen ram semen with storage for around 2 months and 2-10 days respectively.

Failure to obtain any conceptions out of 28 ewes inseminated is mainly attributable to the poor viability of the spermatozoa, as assessed by motility. Good progressive motility is essential for fertility according to Lightfoot and Salamon (1970); Kareta et al. (1971) and Cassou (1972). The percentage of motile spermatozoa here was close to the minimum requirement of 30-40% according to Kareta et al. (1972b) and Colas and Guerin (1974) but well below the standard of around 70% claimed necessary by First, Sevinge and Henneman (1959).

Spermatozoal morphological damage has been shown in earlier experiments to be caused by freezing and to increase following in vitro storage. It also occurs in vivo as shown by Bedford (1963b, 1964a, 1968, 1969 & 1972). Such damage leads to rapid loss in motility (Paufler, 1972; Lindemann and Rikmenspoel, 1972 and Mann and Lutwak-Mann, 1975).

Fertility depends mostly on normal motile spermatozoa according to Smirnov (1953); Hulet et al. (1965) and Varnavskii and Turbin (1974a). On the other hand in the case of ram semen, evaluation on the basis of morphological changes was considered more accurate than motility evaluation by Smorag (1971) and Smorag and Kareta (1974).

It has also been shown in earlier experiments that the thawed spermatozoa have a short life span of less than 6 hours at body temperature, and this may also apply in the genital tract of the ewe. It was hoped that the delay in insemination for 24 hours, and the repeat insemination after 12 hours would enhance the probability of live spermatozoa being present in the oviduct at the time of ovulation. However, as insemination was thus done at about 30 hours and 42 hours after the onset of oestrus, this could possibly be too late, especially if the sperm behaved differently in the female reproductive tract, or the proportion of teaser tups to ewes was too low.

A simple insemination technique, suitable for routine use was employed. It is known that the cervix itself is an obstacle to the passage of spermatozoa weakened by freezing (Loginova, 1962 and Mattner et al. (1969), which in turn increases the rate of spermatozoal death (Loginova and Zheltobryukh, 1972a & b, and Hawk and Conley, 1975a). The lifespan of ram spermatozoa is short, around 9 hours, according to Quinlan, Mare and Roux (1933) and Green and Winters (1935) or less in the case of frozen semen according to Mattner et al. (1969) and Loginova and Zheltobryukh (1974). Moreover, Mann and Lutwak-Mann (1975) found that frozen semen lost fertilizing ability before the cessation of motility, due to the loss of enzyme and co-enzyme sources (as described by Lindemann and Rikmenspoel (1972). Vaginal mucus, which was observed in abundance in many of the inseminated ewes is also harmful according to Quinlan et al. (1933) and Hawk and Conley (1975b). The failure in fertility might thus be partially attributable to the inefficiency of the external cervical insemination compared with deep cervical insemination as observed by Kareta et al. (1971); Varnavshii and Turbin (1974a & b) and Linge (1974). Such could only be determined by an extensive series of fertility trials comparing tubal, uterine and cervical insemination.

CONCLUSION

The modification of the freezing process, introduced to avoid unnecessary temperature fluctuations has been found beneficial in reducing the remarkable loss of motility, during the different stages of the freezing process. They had no effect, however, on the losses which continue during prolonged storage at -196°C. Such low temperature storage itself caused a marked deterioration in the semen.

The semen after freezing and storage was of doubtful quality for successful artificial insemination, at least with a simple insemination technique, such as would be desirable for routine use in the field. However, as indicated, other factors probably contributed to the failure of the field trial.

21) OBSERVATIONS ON SEASONAL EFFECTS ON THE CHARACTERISTICS OF RAM SEMEN

INTRODUCTION

The ram does not have the restricted breeding season typical of the ewe, but seasonal variations in semen production and characteristics are evident (Asdell, 1965 and Terrill, 1969).

Sexual activity of the ram tends to be highest in the fall (autumn) and lowest in the winter (Terrill, 1969), and is markedly inferior in spring and summer (Brady and Gildow, 1939; Kojer, 1951 and Mohri et al., 1970).

Ram semen collected during the non breeding (long daylight) season showed poor quality, that is highly susceptible to cold shock, (Misra and Sengupta, 1965), and deterioration in spermatozoal morphology (Starke, 1949; Juma and Dessauky, 1969 and Fraser, 1971a).

Storage or freezing of ram semen as well as that of other species including the bull and goat collected out of the breeding season, especially in summer, has been found unsatisfactory (Salisbury and Flerchinger, 1967a and Kupferschmied, 1972).

The higher the frequency of semen collection in rams the higher is the susceptibility of the spermatozoa to cold shock (Lasley and Bogart, 1944 and Salamon and Lightfoot, 1967) and the lower the survival rate after deep freezing (Salamon and Lightfoot, 1967 . and Entwistle and Martin, 1972).

On the other hand excessive acrosomal damage has been observed to be associated with sexual rest in the bull (Wells and Awa, 1970b, Wells et al., 1970 & 1971) and in the rabbit by Bedford,(1964a).

Semen, collected by electro-ejaculation did not freeze so well as semen collected by the artificial vagina due to the increased quantities of accessory fluid as observed in rams by Fraser (1968); Quinn et al. (1968a); Visser (1969) and Entwistle and Martin (1972a) as well as in bulls by Colleary and Ehlers (1964).

The aim of the observations recorded here was to obtain an overall picture of the ram semen employed throughout the experimental work and to investigate the effect of season on the semen characteristics.

MATERIALS AND METHODS

Semen collection and evaluation have already been described in the section on general materials and methods.

Over a period of 20 months from March 1973 to October 1974 a total of 114 electro-ejaculation procedures was applied to a total of 14 rams comprising 4 Dorset, 8 Suffolk, one Cheviot and one Border Leicester.

The frequency of collection depended on the individual ram response. Normally it was weekly, rarely twice weekly, but when collection was unsatisfactory, the next attempt at collection was delayed for a fortnight. The most frequently used rams were Cheviot C18 and Dorset D2, which contributed in each case over a period of one year, 25 and 28 respectively of the semen samples evaluated and used in freezing experiments. The remaining rams were used or were available for limited periods only, contributing a range of one to seven samples.

RESULTS

Out of 114 attempted semen collections by electro-ejaculation 80 were successful in yielding samples suitable for evaluation and processing and 34 were unsuccessful in that no sample was obtained, or the sample was contaminated by urine or was watery and azoospermic or contained few or agglutinated spermatozoa only. The distribution of these by ram and month of year is shown in table 107. Dorset ram No. 1 was classed as infertile after two attempted collections and Suffolk C38 after 8 unsatisfactory collections out of 13 attempts. Cheviot ram C18 developed pneumonia in October 1973 and became temporarily infertile giving poor quality semen over the following 3 months.

The distribution of the same 114 results by season is shown in table 108, Spring being defined as February to April, Summer as May to July, Autumn as August to October and Winter as November to January. These results show that there was no seasonal effect on the relative proportions of successful and unsuccessful collections. (x^2=0.51; Degrees of Freedom = 3; $P < 0.9$). Table 109 shows the same data omitting all the results for rams Dorset 1 and Suffolk C38 and the results from Cheviot C18 for 3 months from mid-October. Again there were no significant differences between seasons (x^2 = 0.80; Degrees of Freedom = 3; $P < 0.75$), although relatively fewer collections were unsuccessful in autumn. 75 of the 80 raw samples collected were fully evaluated before processing except that in 4 samples acrosomal detachments and acrosomal damage were not scored separately, and total acrosomal defects only were recorded. The mean values of each parameter for each season of the year are presented in table 110, and the analyses

of variance of the results are presented in appendix table A101. Where analysis of variance showed the existence of a significant seasonal effect, the means were compared for significant differences using students t-test. There were no significant differences between seasons in respect of volume, density or morphological defects other than damage to acrosomes and the total of acrosomal defects. Motility as measured by both mass activity and percentage of motile spermatozoa was highest in summer. Mass activity was significantly greater in summer than in winter or spring while the percentage of motile spermatozoa was significantly higher in summer and autumn than in winter. The percentage of live spermatozoa was significantly higher in autumn than in all other seasons. Total acrosomal defects were significantly fewer in autumn and spring than in winter or summer. Acrosomal damage was least in autumn, and highest in winter.

Only one ram, Dorset D2, was available and unaffected by complicating disease over all four seasons.

The mean parameters for this ram, Dorset D2, are given separately in table 111. Analysis of variance of the results is presented in appendix table A102. The results are broadly similar to the overall results in that there were again significant seasonal differences in motility, and the percentages of live spermatozoa and acrosomal defects. The percentage of tail defects differed significantly in addition, being high in winter and spring.

There were no significant differences between parameters for summer and autumn, except in the case of the percentage of acrosomal defects which was higher in summer. In general, better quality semen was obtained in those two seasons than in winter or spring.

The mean parameters for the 11 rams other than D2 are presented in table 112 and analysis of variance of these results in table A103 in the appendix.

The results show that the only significant differences were in respect of the percentage of live spermatozoa, which was higher in autumn than in all other seasons, and the percentage of spermatozoa with acrosomal defects, which was higher in spring than in autumn.

DISCUSSION

Both satisfactory and unsatisfactory distribution of semen collections over the four seasons seemed to be unaffected by season. In other words ram semen could be collected all year round, which is in agreement with the findings of Asdell (1965); Terrill (1969) and Colas, Laszczka, Brice and Ortavant (1972). However, there were fewest unsatisfactory semen collections, and semen quality in general was best, during autumn, and this might be due to the sexual activity of the rams being highest during this season (Brady and Gildow, 1939; Kojer, 1951; Terrill, 1969; Mohri et al., 1970 and Kastyak, 1972). In addition semen samples collected during the other seasons especially summer and winter contained a high percentage of acrosomal defects as also found by Starke (1949); Juma and Dessauky (1969) and Fraser (1971a).

The higher incidence of acrosomal defects is partially explained by the relatively low rate of collection. Ram semen can be collected daily over one month without affecting quality or even fertility according to Salamon (1962 & 1964); Salamon and Lightfoot (1967); Sahni and Roy (1968); Sharma et al. (1968) and Jones (1971a). Here, collection was usually weekly, but rests were given following unsatisfactory collections, of which there were more outwith autumn. Sexual

rest increases the incidence of acrosomal defects in the bull (Wells and Awa, 1970b; Wells et al., 1970 & 1971) and in the rabbit (Bedford, 1964a).

Reference to the raw data for the various experiments indicates that the percentage of spermatozoa with tail defects varied much more between samples than between seasons.

Comparison of the results in tables 110, 111 and 112 show that much of the seasonal variation in semen quality was due to one ram, Dorset D2, which produced much better quality semen in summer than the other rams. It is evident that the latter produced their best semen in autumn, a finding in agreement with those of Terrill (1969) and Kastyak (1972). The Dorset ewe has, of course, an extended breeding season of almost the whole year and it is not surprising to find a similarly extended seasonal effect on a ram of this breed.

CONCLUSION

Most of the various parameters assessed fell within the standard ranges given by other writers who have assessed ram semen collected by electro-ejaculation. However, seasonal effects have been demonstrated to exist, samples being generally better in summer and autumn.

The overall result however was obviously affected by the extensive use of one ram, Dorset D2, a breed with an extended breeding season. When its influence is removed, the results showed fewer seasonal variations, but in autumn, samples still contained the highest percentage of live spermatozoa and fewest acrosomal defects.

THE GENERAL CONCLUSION

The conclusions of this study can be outlined under the following headings:-

1) Semen collection.

2) Stages of the deep freezing process.

3) Modification of the deep freezing process.

1) Semen Collection

Out of the 114 attempted semen collections by electro-ejaculation over 20 months, 80 were successful in yielding samples suitable for evaluation and processing and 34 were unsuccessful in that no samples or poor quality samples were obtained (table 107), due in some cases to the permanent or temporary infertility of the ram.

The distribution by seasons of all 114 collections and of 91 collections, omitting those from infertile rams is shown in tables 108 and 109 respectively. The results showed that there was no significant seasonal effect on the relative proportions of successful and unsuccessful collections. However, various parameters used to assess semen quality were affected by seasons, and in general semen collected during autumn was best.

On the other hand strict comparison of effects of season on rams cannot be done validly because only one ram (Dorset D2) was available and used over all seasons of the year.

2) Stages of the freezing process

A - Dilution

Following dilution of the raw semen spermatozoa showed a little

reduction in their viability and an increase in their acrosomal defects but little change in tail defects.

The period between semen collection and dilution can be considered as an in-vitro storage at 37°C of the raw semen samples which led to the attenuation of the spermatozoa. Moreover, morphological damage especially of the plasma membrane may take place following the mechanical action of dilution itself. The drop in the spermatozoal live percentages after dilution is due to either the death of the spermatozoa as the result of these deleterious factors or an increase in the eosinophilic count after dilution as a result of increased permeability of the plasma membrane of both dead and immobile live spermatozoa.

On the other hand some semen samples became completely immotile after dilution and the cause of that might be due to the sudden change in the spermatozoal environment, agglutination or dilution shock.

B - Equilibration

Following equilibration of the processed semen at 4°C there was a marked decrease in the spermatozoal viability and an increase in the morphological deteriorations. These changes usually increased with length of equilibration. However, the changes might be the result of aging phenomena of the spermatozoa which in turn predispose the spermatozoa to cold shock or weakness. Following damage to the acrosome as well as the plasma membrane of the spermatozoa some degree of lipoprotein loss might be the result which also predisposes the spermatozoa to cold shock.

On the other hand tail defects showed a tendancy to be reduced following equilibration of the diluted semen, probably due to the reduction in the spermatozoal viability. Head and neck defects were very few and their increase following each stage of the freezing process was very slight.

C - Freezing and storage (thawing)

Following the actual freezing on dry ice and storage in liquid nitrogen the reduction in the spermatozoal viability and their increases in the morphological deteriorations became very great especially following thawing after one month's storage.

These massive changes might be due to the high susceptibility of the ram spermatozoa to cold shock or ice crystalization within the spermatozoa during their passage from $4^{o}C$ to $-79^{o}C$. It might in part also be due to temperature fluctuation during filling the containers of frozen pellets as well as on removal of pellets for thawing, during which the whole carrier has to be raised from the liquid nitrogen, especially when the sample is situated at the lower end (figure 8). Such stress increases the morphological deterioration leading to a great loss in the spermatozoal lipoprotein.

These changes in the spermatozoal viability and morphology took place similarly in all the experiments and were not greatly influenced by change of cryoprotective or other modifications (see table 85 and figure 50 which present the overall comparison of glycerol and DMSO in 24 samples each).

Freezability of the raw semen throughout the experimental work, as judged by the spermatozoal viability and morphology was affected by

sample variation even, as in the preliminary experiment, when all samples came from a single ram (Cheviot C18).

3. Modifications

The effect of cryoprotective agents on semen parameters was studied by inclusion of 2-8% glycerol, 2-8% dimethyl sulphoxide (DMSO) and the combination of 4% glycerol with 3% DMSO or 2% glycerol with 1.5% DMSO in an egg yolk and lactose diluent.

4% glycerol was the least deleterious to the spermatozoal viability throughout the freezing process. 2% glycerol seemed to be insufficient to provide protection to the spermatozoa during freezing. On the other hand 6% and especially 8% glycerol were toxic. Moreover, acrosomal defects increased as the glycerol level increased from 2 - 8%.

DMSO gave similar results except that 2 and 4% did not differ much in protecting the spermatozoa during freezing. Therefore, a compromise of 3% was taken as the optimum.

On the other hand the comparison between 4% glycerol and 3% DMSO showed that glycerol was less toxic to the spermatozoa than DMSO. In addition, in an overall comparison between the effects of 4% glycerol and 3% DMSO on 24 analogous samples (table 85 and figure 50), the results showed little difference during storage at 4°C, but the post-thawing activity was better in the case of glycerol. Morphological defects seemed to be affected similarly by both cryoprotectives. Glycerol however, was found to interfere with the eosin differential staining for assessment of live spermatozoa more than DMSO.

The protective efficiency of 4% glycerol and a combination of 2% glycerol with 1.5% DMSO was nearly the same, but 4% glycerol was still superior. Egg yolk and lactose alone protected ram spermatozoa during freezing to some extent as judged by their post-thawing motility. On the other hand live percentages did not differ from those with cryoprotectives. However, it can be concluded that the assessment of freezing efficiency has to be judged by motility, and morphology only because the live percentage seemed to be unreliable.

Increasing the egg yolk level from 25% to 50% with or without sodium citrate in 4% glycerol containing diluents was harmful which might be the result of binding up of glycerol.

Various equilibration times (0.5 - 24.0 hours), dilution rates (1:1 - 1:10), thawing media (sodium chloride, sodium citrate with or without lactose, in state of solution at 37°C or frozen pellets at -196°C) and thawing temperatures (0°C - 100°C) in 4% glycerol or 3% DMSO containing diluents were tried.

The optimum duration of equilibration was 3 hours in the case of glycerol and 1.5 hours in the case of DMSO. This difference might be due to extra-cellular protection of the DMSO or the faster penetration of the spermatozoa by DMSO. However, equilibration for 1.5 hours in case of glycerol was also sufficient. On the other hand 0.5 hours equilibration was not sufficient for either glycerol or DMSO to penetrate the cell membranes. Overnight equilibration was deleterious especially in the case of glycerol.

Dilution rate of 1:4 was optimum irrespective of the cryoprotective. Lower dilution rates especially 1:1 were harmful which might be

due to the inactivation of the spermatozoa as a result of low pH and accumulation of lactic acid especially in lactose containing diluents, which is not neutralized by the relatively small amount of buffer in egg yolk. In addition low dilution rate lowered the final level of the cryoprotective. On the other hand dilution rates higher than 1:6 were also harmful and associated with morphological defects as well as predisposing ram spermatozoa to cold shock during cooling and freezing.

Thawing of the frozen pellets in a dry test tube was better than thawing involving use of a thawing medium. Redilution of the thawed semen with any thawing media seemed to be harmful. However, thawing media in a state of warmed solution at 37°C were superior to those in a state of frozen pellets at -196°C.

Fast thawing at 37°C to 100°C was superior to slow thawing at 0°C and especially 20°C. However, thawing at body temperature (37°C-39°C) is preferrable, giving reasonably fast thawing without the risk of over-heating the spermatozoa themselves.

Various methods of dilution, i.e. slow, by adding the diluent drop by drop (figure 6) and fast by direct addition at both 4°C and 20°C or their combination have been tried as well. The results showed that equilibration at 20°C irrespective of method of dilution provide surviving spermatozoa some resistance to cold shock during freezing of the semen, but most of the spermatozoa were killed by such treatment. On the other hand there was little difference between methods of addition of the diluent irrespective of the equilibration temperature. A combination of the methods by addition of the diluent

by dropping for 0.5 hour at 20°C followed by 1.0 hour storage at 4°C gave slightly improved results.

Prompt dilution of the raw semen and direct dropping of the frozen pellets from the dry ice to the liquid nitrogen for storage, as well as extra care in the sampling of the frozen pellets for thawing purposes (Experiment No. 16) led to a marked improvement in motility on thawing after 24 hours storage. However, it did not prevent the loss which occurs on longer storage.

The post-thawing life span of the ram spermatozoa at 39°C was short, around 3 hours, and this would almost certainly also apply in the genital tract of the ewe. Acrosomal defects increased progressively as the incubation time at 39°C increased from 0 to 3 hours which was probably associated with loss of their phospholipids and proteins.

The post-thawing motility did not exceed 40%, whereas 70% motile spermatozoa are considered necessary for satisfactory results in sheep artificial insemination.

In addition, morphological soundness of the spermatozoa, especially their acrosomes, is necessary for fertilization.

Study of the spermatozoal morphology under the electron microscope showed that the morphological damage which occurred in the acrosome seemed to be a series of changes from the fracture of the plasma membrane to the complete detachment of the whole acrosome leaving the nucleus denuded (figures 59-68). These changes are similar to those of the acrosomal reaction during capacitation.

Moreover, the actual site of the freezing damage were both the acrosome and the midpiece (figure 70) especially the mitochondria.

These morphological changes have been observed in the raw as well as processed semen. Although the damage could not be ascertained quantitatively by stages, it seemed that its severity increased following each stage of the freezing process.

The unsatisfactory result of the insemination trials was probably due to the short life span of the relatively few spermatozoa which survived freezing and thawing. Moreover semen is deposited on or near the cervical os, following simple insemination technique. It is known that the cervix itself is an obstacle to the passage of spermatozoa weakened by freezing, which in turn increases the rate of spermatozoal death. Vaginal mucus, which was observed in abundance in many of the inseminated ewes can also be harmful to the spermatozoa. The importance of these and other factors can only be ascertained by extensive fertility trials which are outside the remit of this thesis.

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